Safety of herbal preparations on the Dutch market

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ABSTRACT

The use and availability of herbal preparations covered by food law is increasing in the Netherlands and in other European Member States. Correspondingly, safety concerns relating to herbal preparations are growing as well. The aim of the present PhD project was therefore to review the toxicity of selected herbal preparations, to investigate the presence and actual levels of selected naturally-occurring toxic substances and contaminants in herbal preparations on the Dutch market and to estimate the associated risks.

First, an overview is provided of the Dutch and European legal provisions for food commodities with botanical ingredients, the nature and mechanism of action of various toxic botanical ingredients specifically covered by these provisions, and the health concerns defined by risk assessors related to several botanicals for which no specific legal provisions exist. Secondly, data are presented on the actual occurrence in traditional herbal preparations (THPs) of a group of phytotoxins, i.e. aristolochic acids, which were banned by the Dutch Commodities Act Decree 'Herbal preparations'. Aristolochic acids and derivatives are nephrotoxic, genotoxic and carcinogenic and are present in several plants from the *Aristolochiaceae* family. Aristolochic acids were found in 25 of 190 THPs used in Traditional Chinese Medicine (TCM) sampled on the Dutch market. This shows that testing for aristolochic acids of Chinese THPs at risk of contamination is essential in the framework of food safety.

Thirdly, the presence of selected toxic contaminants in herbal preparations on the Dutch market was investigated. Lead, mercury and arsenic levels were analyzed in THPs used in several Asian traditional medicine systems, such as Ayurveda, TCM, and Traditional Tibetan Medicine (TTM). These metals and metalloids were present in 186 (64%) of 292 THPs and use at recommended dose levels of 59 THPs (20%) would result in intakes of these contaminants significantly above established toxicological safety limits. It was concluded that the mercury, arsenic and lead contents of these Asian THPs are cause for concern. Because metals such as mercury can exist in various defined chemical species with different toxic properties, a study was performed using selective acid extraction procedures to determine the presence or absence of the relatively non-toxic elemental form of mercury in 19 Ayurvedic THPs, which were shown in the previous study to result in mercury intakes above the safety limit for inorganic mercury when used at the recommended daily dose level. It was concluded that in these THPs the main part of the mercury content is not present in the elemental form, that the mercury detected in Ayurvedic THPs is likely to be present in the inorganic form and that therefore the estimation of the related risks based on the safety limits for inorganic mercury is justified.

In the last study of this PhD thesis, polycyclic aromatic hydrocarbons (PAH) were determined in more than 1500 food supplements sampled on the Dutch market, many of which contained herbal ingredients. Herbal preparations can become contaminated with PAH through various processes including direct atmospheric deposition on plant surfaces and drying practices during manufacturing. Several PAH, such as benzo[a]pyrene are genotoxic and carcinogenic. Supplements containing herbal ingredients such as St. John's wort and *Ginkgo biloba*, the phytochemical resveratrol and the bee product propolis showed the highest mean PAH levels. It was shown that individual food supplements can contribute significantly to PAH exposure, whereas on average PAH

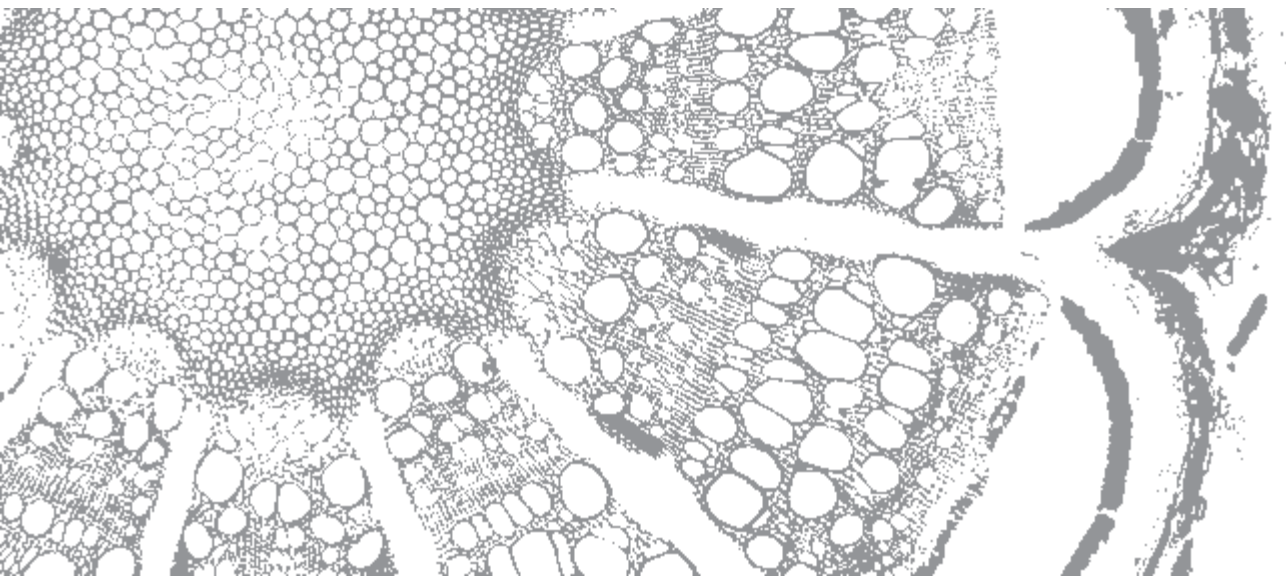
intake resulting from food supplement use will be at the lower end of the range of contributions of main food groups to PAH exposure.

From the work described in this thesis it can be concluded that for herbal preparations ‘natural’ does not equal ‘safe’. Given that uncertainty exists whether additional European legal measures will be taken in the near future to restrict or prohibit the use of specific toxic herbal substances in foods and the fact that several herbal preparations for which specific provisions are absent in Dutch food safety law raise toxicological concern, would suggest that it is prudent to keep the Dutch Decree ‘Herbal preparations’ and other national legislation up to date in order to protect consumers from serious risks resulting from use of botanicals in food products such as herbal preparations.

CONTENTS

CHAPTER
1

General introduction



Introduction

This PhD thesis focuses on the safety of selected herbal preparations on the Dutch market. It concerns exclusively herbal preparations that are used orally and which are regulated by Dutch food law. Because a significant part of the research included in the project involves official controls by the Voedsel en Waren Autoriteit (VWA) – the Dutch Food and Consumer Safety Authority – safety concerns regarding herbal preparations are also approached from a legal perspective. The European Food Safety Authority (EFSA) recognized in 2004 that a large number of botanical materials and botanical preparations obtained from these materials by various processes were finding their way onto the food supplement market and that safety concerns existed for several of these products. The safety concerns related to botanicals and botanical preparations essentially relate to i) the presence of naturally-occurring toxic substances in plants, ii) the intentional addition or accidental occurrence of toxic contaminants and iii) interactions of botanical ingredients with active ingredients from medicinal products (herbal food-drug interactions) [1]. In the Netherlands, safety concerns related to specific herbal preparations including several issues referred to by EFSA such as the presence of toxic pyrrolizidine alkaloids or aristolochic acids in herbal preparations were addressed in 2001 by the Commodities Act Decree 'Herbal preparations' [1, 2]. Furthermore, the Decree prohibits the placing on the market of any herbal preparation that contains herbal substances in amounts that are detrimental to health [2].

The aim of the present PhD project was to review the toxicity of selected herbal preparations, to investigate the presence and actual levels of selected contaminants and naturally-occurring toxic substances in herbal preparations on the Dutch market and to estimate the associated risks.

Legal framework for herbal preparations and related botanical products

The Dutch Commodities Act and its subordinate Decrees and Directives fall under the responsibility of the Ministry of Health. The Act regulates foods and a wide array of other commodities. The Commodities Act Decree 'Herbal preparations' (in Dutch: 'het Warenwetbesluit Kruidenpreparaten') covers herbal preparations that are brought on the market as foods and non-food products [2]. The Decree defines herbal preparations as 'herbal substances, subjected to treatment or not, including herbal extracts, which are intended to be used by humans'. In addition, 'herbal substances' are defined as 'substances that are composed of plant material'. According to EFSA, the term 'botanical' includes all botanical materials (e.g. whole, fragmented or cut plants, plant parts, algae, fungi and lichens) and the term 'botanical preparation' includes all preparations obtained from botanicals by various processes (e.g. pressing, squeezing, extraction, fractionation, distillation, concentration, drying up and fermentation) [3]. The terms 'botanical material' and 'botanical preparation' used by EFSA are essentially synonyms of respectively the terms 'herbal substance' and 'herbal preparation' as defined by the Decree 'Herbal preparations'. However, the terms used by EFSA are not defined by law and are not linked to any legal category. In the current thesis the term 'botanical' can refer to either a botanical material or a botanical preparation as defined by EFSA without implying a certain legal category. Most botanicals for oral use on the Dutch market are herbal preparations covered by the Decree 'Herbal preparations' and the minority is regulated as medicinal

products or is covered by other legislation. Recent European legislation established traditional herbal medicinal products as a new category of medicinal products for human use [4]. This new Community legislation includes amongst others provisions for the establishment of the Committee for Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA; at that time the acronym EMEA was used) and this legislation has been reviewed elsewhere [5]. Products from this new category share several characteristics with herbal preparations such as botanical food supplements. An important difference is however that within European Member States, traditional herbal medicinal products can only be brought on the market after an authorization, whereas market introduction of botanical food supplements at present does not require a safety assessment and market authorization.

European legislation applicable to food supplements with botanical ingredients

Most herbal preparations covered by the present PhD project are food supplements with botanical ingredients. European Directive 2002/46/EC covers food supplements which, according to the preamble to this directive, can not only contain vitamins and minerals but also a wide range of other ingredients including various plants and herbal extracts [6]. The directive does however not specify which plants or herbal extracts can be used for the manufacture of food supplements. Moreover, European food law does not include any other specific legislation on the use of substances other than vitamins or minerals in food supplements similar to the Dutch Commodities Act Decree 'Herbal preparations', which only applies to the Netherlands. In the European legislative context, the use of plants or herbal extracts in food supplements is therefore subject to general rules relating to food safety [4]. Regulation (EC) No 178/2002, also called the General Food Law, which aims to harmonize food safety legislation in European Member States, includes such general rules. For example, the General Food Law explicitly forbids the placing of foods on the market, which are injurious to health or unfit for human consumption [7]. Furthermore, the General Food Law lays the primary legal responsibility for the safety of the food products on the business operators who placed these products on the market. The General Food Law does not provide any guidance on how the safety of foods should be assessed. To fill in this gap for botanical food supplements, EFSA developed a two-level tiered approach for the safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements [3], which was tested by an EFSA Scientific Cooperation (ESCO) Working Group composed of experts from Member States [8]. The final version of the resulting Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements was published in 2009 [3]. Furthermore, a Compendium of botanicals reported to contain toxic, addictive, psychotropic, or other substances of concern was compiled by EFSA to be used as a preliminary tool for risk assessors. The Compendium was validated by the ESCO working group and the final version was published in 2009 together with the Guidance document [9]. The Compendium has no legal status, but aims at flagging plants or parts of plants or substances of possible concern for human heath naturally present in the listed botanicals. The Compendium is partly based on existing national lists of botanicals that are restricted or not admitted in food in several Member States [3].

EFSA advised that priority for risk assessments should be given to those plants i) that are known to have an established history of food use and contain significant levels of substances of concern, ii) that are not allowed or recommended for food use in some European countries, iii) for which some adverse health effects have been reported, iv) for which consumption has significantly increased during recent years and for which the intended use levels are expected to be relatively high and v) for which there are both limited history of use and toxicity data available [3]. A more detailed overview of the current Dutch and European regulatory framework for selected food commodities with botanical ingredients is presented in Chapter 2 of this thesis.

Plants with toxic constituents

Plants do not only produce carbohydrates, fats or proteins, which are called primary metabolites. Another group called secondary metabolites includes a multitude of compounds such as alkaloids, polyphenols, coumarins, carotenoids, and other compounds that can exert a physiological effect in humans [10]. Any efficacy shown by botanical products is usually the result of the effects of one or more plant secondary metabolites and much effort is directed at identifying these compounds, which are also called active constituents [1, 11]. For many botanicals the active constituents have been identified but for others it has been proven hard to identify the compounds responsible for the effect [3]. In addition to their perceived beneficial effects on health, botanical constituents can also prove to be toxic causing adverse health effects. As a result, herbal preparations can be associated with direct and indirect health risks. An important example of an indirect health risk is interaction with conventional medicines, which will be discussed in some detail further on in this chapter. Furthermore, there are several types of direct toxic effects. De Smet [12] recognized the following categories of direct adverse reactions to herbal preparations: i) pharmacologically predictable and usually dose-dependent reactions, ii) idiosyncratic reactions, which are unpredictable and do not show a correlation between dose level and toxicological risk, iii) reactions that develop during long-term therapy and iv) delayed effects such as carcinogenicity and teratogenicity. In Chapter 3 of the present thesis, the nature and mechanism of action of several toxic botanical ingredients will be discussed. These botanicals are, or will probably be, specifically covered by Dutch or European legislation and are presently receiving increased attention in the field of food toxicology.

Aristolochia spp. are an example of herbs with delayed toxic effects. It was determined that these effects were linked to naturally-occurring constituents of these plants called aristolochic acids. In Traditional Chinese Medicine (TCM), *Aristolochia* spp. are used in several traditional herbal preparations. Several species from the genera *Aristolochia* and *Asarum*, which belong to the family of the *Aristolochaceae*, contain aristolochic acids and related compounds. The kidney is an important target of aristolochic acids and their toxic action can result in renal failure. In the early 1990s, female patients of a Belgian slimming clinic were prescribed herbal preparations contaminated with aristolochic acids and subsequently developed rapidly progressing renal interstitial fibrosis, later referred to as aristolochic acid nephropathy (AAN). Furthermore, aristolochic acids and derivatives are both genotoxic and carcinogenic. Several of the Belgian patients diagnosed with AAN also developed urothelial cancer [13]. As a result, herbs with aristolochic acids were prohibited in the Netherlands in 2001 and other in countries

worldwide. The toxicity of aristolochic acids is discussed in Chapters 3 and 4 of this thesis and the occurrence of herbal preparations with aristolochic acids on the Dutch market is explored in Chapter 4 of this thesis.

Contamination of herbal preparations

Contamination is one of the safety issues recognized by EFSA to be relevant to herbal preparations [1]. This contamination can be either deliberate or unintentionally. Adulteration of herbal preparations with toxic herbs in Asian traditional medicine is a recognized problem. A cause of adulteration can be confusion over names. This has for example been reported to occur for plants that in Traditional Chinese Medicine (TCM) are used interchangeably with *Aristolochia* species (discussed in Chapter 4). Contamination of herbal preparations with a wide range of other substances than toxic plant material has been described. Contaminants include metals and metalloids such as lead, mercury and arsenic, synthetic drugs, polycyclic aromatic hydrocarbons (PAH) and other undesirable substances [1, 14, 15]. Synthetic drugs such as sibutramine or illegal analogs of sildenafil which is the active substance of Viagra® are relatively frequently found in botanical food supplements sampled by the VWA and other Dutch governmental services on the Dutch market. These adulterations are of major toxicological concern and the related health risks have increased in the last decade [14, 16, 17]. Especially botanical products originating from Asia are frequently contaminated with synthetic drugs, and therefore the growing volume of sales in the European Union for traditional herbal products (THPs) obtained from suppliers based in Asia and the increase in the number of outlets of products of traditional medicine are cause for concern [1]. Traditional medicine is defined by the World Health Organization (WHO) as: “the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses”. The terms complementary-, alternative-, non-conventional medicine are used interchangeably with traditional medicine [18].

Intentional addition of metals and metalloids to THPs has been described to occur in several Asian traditional medicine systems [14]. In the Indian traditional medicine system Ayurveda, Traditional Tibetan Medicine (TTM) and in TCM, the metals mercury and lead and the metalloid arsenic are deliberately added to THPs for therapeutic reasons. This practice has caused poisonings with Asian THPs worldwide. In Chapter 5 of this thesis the occurrence of these metals and metalloids in Asian THPs on the Dutch market is investigated and the related risks are assessed. Metals and metalloids can exist in different defined chemical species and the toxicity of these species can vary significantly (WHO, 2006). In order to estimate the risks related to exposure to metals and metalloids from food it is important to take into account the chemical species in which these metals and metalloids exist. This is illustrated in the example of mercury. The main types of mercury species are elemental mercury, inorganic and organic mercury compounds. Mercury is present in inorganic compounds as monovalent or divalent mercury. Organic mercury results from the combination of carbon and mercury and methylmercury is a highly toxic organic mercury species that can diffuse through phospholipid membranes [19, 20]. Inorganic mercury is nephrotoxic and neurotoxic and long-term use of preparations with inorganic mercury compounds has resulted in poisonings worldwide [21]. In contrast, elemental mercury shows a low oral toxicity

when compared to organic and inorganic mercury compounds [19, 21]. This finding is especially relevant for mercury exposure from Ayurvedic THPs. In Ayurveda, elemental mercury is used as a starting material for mercury based ingredients that are an integral part of several Ayurvedic THPs. The elemental mercury is however extensively processed and mixed with sulfur and herbs before the resulting mercury based ingredient is added to Ayurvedic THPs. It is likely that during the preparation of Ayurvedic THPs the elemental mercury is completely transformed into inorganic mercury compounds, but conclusive analytical data supporting or rejecting this assumption is lacking. Because elemental mercury shows a low oral toxicity, a relative high elemental mercury level would require another type of safety limits than the safety limits for inorganic mercury used in Chapter 5. Thus, an important aspect in the risk assessment of mercury from the Ayurvedic THPs is whether mercury in these THPs is present as elemental mercury or not. A study into the presence or absence of the non-toxic elemental form of mercury in Ayurvedic THPs was therefore conducted and the results of this study are presented in Chapter 6.

As opposed to deliberate contamination of Asian herbal preparations with metals, metalloids or synthetic drugs, another group of contaminants are introduced unintentionally from the environment or through processing. Examples of this last group that are also found in herbal preparations are polycyclic aromatic hydrocarbons (PAH). These substances form a large class of organic compounds that are composed of two or more fused aromatic rings. PAH can occur in foods as a result from various processes. PAH can contaminate foods during smoking processes and heating and drying processes that allow combustion products to come into direct contact with the food. In addition, environmental pollution may cause contamination with PAH. These environmental PAH may be concentrated by the extraction steps applied in the production process of the botanical preparation, resulting in the past in for instance vitamin E samples that were highly PAH contaminated [15, 22-24]. Several PAH, such as benzo[a]pyrene are genotoxic and carcinogenic. PAH were evaluated by several risk assessment bodies such as the Dutch National Institute for Public Health and the Environment – het Rijksinstituut voor Volksgezondheid en Milieu (RIVM), the Scientific Committee on Food (SCF) of the European Union (EU) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [25-27]. In 2008, the European Food Safety Authority (EFSA), which continued the work of the SCF, reviewed the available data on occurrence in food and toxicity of 16 priority PAH, 15 of which were identified by SCF and one, benzo[c]fluorene, by JECFA. EFSA concluded that 8 of these 16 'EFSA priority PAH' for which oral carcinogenicity data are available (PAH8) could be used as indicator of the carcinogenic potency of PAH in food. EFSA concluded that the most suitable indicators of PAH in food with regards to both occurrence and toxicity were currently a combination of 4 compounds of the PAH8 (referred to as PAH4) or PAH8 itself, with PAH8 not providing much added value compared to PAH4 [28]. The occurrence and connected risk assessment of PAH in herbal preparations on the Dutch market is explored in Chapter 7 of this thesis.

Interactions of botanicals with medicinal products

An indirect health risk of herbal preparations is the potential of certain herbs such as St. John's wort (*Hypericum perforatum* L.) to interact with medicinal products [1]. The potential of St. John's wort to interact with

certain medicines is stated in the package leaflets that accompany those medicines. EFSA noted however that contra-indications do not usually appear on the labels or any associated documentation when botanical food products are sold through supermarkets and other retail outlets. Furthermore, EFSA cited a study from 2001 by Izzo and Ernst who reviewed the seven top-selling herbal products (ginkgo, St. John's wort, ginseng, garlic, echinacea, saw palmetto and kava) and recorded adverse drug interactions for Ginkgo, garlic, St. John's wort, kava and ginseng but not for echinacea and saw palmetto [1, 29]. Interactions of herbal products with prescription drugs received increased attention in 1999 following a review published in the Lancet on the safety of St. John's wort when used concomitantly with various prescribed drugs [30]. These prescribed drugs were drugs that are metabolized by hepatic cytochrome P450 (CYP) enzymes which activities could be doubled by St. John's wort possibly leading to sub-therapeutic plasma concentrations of the drugs [30]. Subsequently, the EMA issued a warning for this potential effect of St. John's wort in February 2000 [31]. HMPC of EMA adopted in November 2009 a Community herbal monograph on *Hypericum perforatum* L., herba (well-established medicinal use)(St. John's wort), in which it is established that concomitant use with cyclosporine, tacrolimus for systemic use, amprenavir, indinavir and other protease inhibitors, irinotecan and warfarin is contraindicated. The monograph also defines other cautions related to interactions with medicinal products including the warning that women using oral contraceptives should take additional contraceptive measures [32]. EMA also considered other possible herb-drug interactions, such as interactions between botanical laxative bulk producers as psyllium seed, ispaghula seed and husk and linseed, and certain medicines. EMA recommended to include a statement on the labeling of these botanical laxatives on the risk of interaction with medicinal products against diarrhea such as loperamide [33]. Because of the potential of St. John's wort to interact with a wide range of drugs and the severity of several of these herb-drug interactions, the mechanism of action is discussed in more detail in Chapter 3 of this thesis. The topic of herb-drug interactions will however not be discussed further in this thesis.

Objective and outline of this thesis

The aim of the present PhD project was to review the toxicity of selected herbal preparations, to investigate the presence and actual levels of selected contaminants and naturally-occurring toxic substances in herbal preparations on the Dutch market and to estimate the associated risks. Three categories of safety issues related to herbal preparations have been identified by EFSA including i) the presence of naturally occurring toxic substances in plants, ii) contamination, and iii) interactions with medicinal products [1]. In this thesis mainly safety concerns of the first two categories were investigated.

Chapter 1, the present chapter, provides a general introduction to safety concerns related to herbal preparations and other botanical products that are brought on the market as food commodities in the Netherlands. It also provides a short introduction on legislation and regulation relating to botanical products intended to be ingested by humans. **Chapter 2** presents a synopsis of safety issues relating to botanicals that are

bodies on the risks of selected botanicals currently not covered by the Dutch Commodities Act are briefly discussed. **Chapter 3** describes the nature and mechanism of action of several naturally occurring toxic substances in plants introduced in Chapter 2. **Chapter 4** discusses the results of investigations into the occurrence of aristolochic acids in herbal preparations used in TCM sampled on the Dutch market and the risks associated with exposure to aristolochic acids from these specific Chinese traditional herbal preparations. **Chapter 5** focuses on the contamination of herbal preparations on the Dutch market that are used in the Indian traditional medicine system Ayurveda, Traditional Tibetan Medicine (TTM) and TCM with mercury, lead and arsenic, and assesses the related risks by comparing the estimated heavy metal intake resulting from use at the proposed dose levels to established toxicological safety limits. In **Chapter 6** it is investigated whether mercury found in Ayurvedic THPs was present as the non-toxic elemental mercury or not, in order to facilitate and further support the selection of established toxicological safety limits to be used for risk assessment providing the basis for subsequent market interventions. To this end, a method enabling detection of elemental mercury in the Ayurvedic THPs was established followed by detection of the possible presence of elemental mercury. **Chapter 7** presents data obtained in a survey into the occurrence of genotoxic and carcinogenic PAH in different food supplement categories. Because it was recognized from data obtained in 2003, the first year of the survey, that high benzo[a]pyrene levels are frequent in botanical food supplements special emphasis was placed on this category. Furthermore, the analytical method was expanded to include more of the 16 priority PAH defined by EFSA in 2008 in addition to benzo[a]pyrene. Finally, **Chapter 8** 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 herbal preparations currently on the Dutch market and what recommendations can be provided regarding the regulatory framework for herbal preparations on the basis of these findings.

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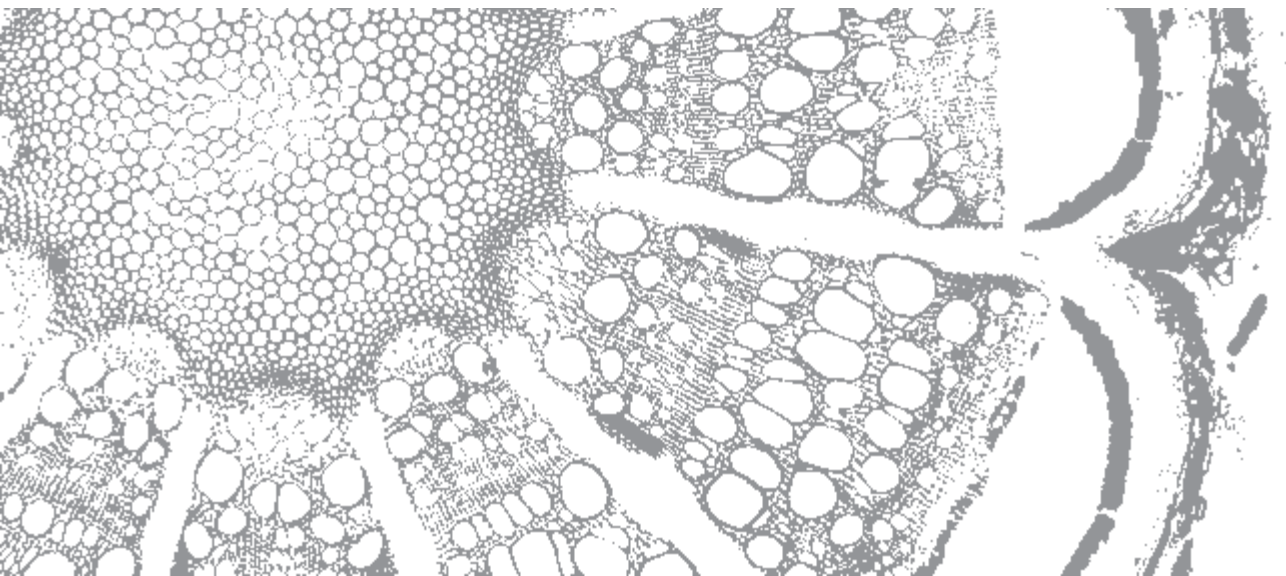
CHAPTER
2

The Dutch and European regulatory framework for selected food commodities with botanical ingredients



Abstract

In the Netherlands, the Commodities Act Decree 'Herbal preparations' regulates the use of toxic plants and fungi in herbal preparations. It includes limits for three types of phytotoxins in herbal preparations, which are toxic pyrrolizidine alkaloids, aristolochic acids and yohimbe alkaloids. Furthermore, the Decree currently prohibits the use in herbal preparations of 46 plants and fungi that are too toxic to be used in food or in other commodities. The constituents responsible for the toxic effects of these 46 plants are also found in plants not specifically regulated by the Decree. Within Europe, EFSA developed a new methodology for the safety assessment of botanicals used in food supplements and published a Compendium of botanicals that raise toxicological concern to be used as input for risk assessments. European and Dutch risk assessment bodies concluded that several botanicals pose health risks. The current paper presents an overview of the Dutch and European regulatory framework for selected food commodities with botanical ingredients as well as an overview of health concerns defined by risk assessors related to botanicals not specifically covered by these food safety laws. It is concluded that the current regulation and legislation in the Netherlands and Europe needs to be updated to address health risks relating to botanicals and botanical preparations that are identified to be of concern.

Introduction

In the Netherlands, the Commodities Act and its subordinate Decrees and Directives regulate foods and a wide array of other commodities. The Dutch Commodities Act Decree 'Herbal preparations' (in Dutch: 'het Warenwetbesluit Kruidenpreparaten') covers herbal preparations that are brought on the market as foods (often as food supplements) and non-food commodities [1]. European food law does not include specific provisions that determine which botanicals can or cannot be used in food supplements. Within the European regulatory framework, the use of botanicals in food supplements is therefore subject to general rules in European food safety law including general rules established by Regulation (EC) No 178/2002. This Regulation is called the General Food Law and it explicitly forbids the placing of foods on the market, which are injurious to health or unfit for human consumption [2]. EFSA developed a two-level tiered approach for the safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements [3]. This method can then be used to assess whether a particular botanical is injurious to health or unfit for human consumption. Furthermore, a Compendium of botanicals reported to contain toxic, addictive, psychotropic, or other substances of concern was established by EFSA to be used as a preliminary tool for risk assessors. The Compendium is based in part on existing national lists of botanicals that are restricted or not admitted in food in several Member States [3]. A list of botanicals that are too toxic to be used in food or in other commodities included in the Dutch Decree 'Herbal preparations' was also used to compile the Compendium. European food safety legislation does contain provisions for several plant toxins in foods other than food supplements, and it also establishes maximum levels for certain contaminants of botanicals used in various categories of food products including food supplements. In the Netherlands, these European provisions have been transposed to the Dutch Commodities Act.

The current Chapter will discuss selected safety issues relating to the use of botanicals in food, which are currently addressed by Dutch and European food legislation and which are of relevance to this PhD project. It is however not an in-depth review of the regulatory framework for botanical products that are intended to be consumed by humans. It will for instance only briefly touch upon legislation for medicinal products. The Chapter will focus on plants with toxic constituents regulated by the Dutch Commodities Act Decree 'Herbal preparations', but also on plants not regulated by the Decree but with identical or related toxic constituents. To this latter end, the scientific opinions of European and national risk assessment bodies on the risks of selected botanicals relevant to the Dutch market but currently not specifically covered by the Dutch Commodities Act will be discussed. The molecular mechanisms of toxicity of several of the phytotoxins for which legal provisions have been introduced in European and Dutch food law will then be discussed in more detail in Chapter 3 of this thesis. The present Chapter will also present information on the regulation and legislation for contaminants that may occur in botanical preparations, including toxic herbal adulterants, synthetic drugs, metals, mycotoxins and polycyclic aromatic hydrocarbons (PAH). Finally, the Chapter presents some considerations on products on the borderline with other legislation, notably the legislation for medicinal products and associated classification issues, and on the use of botanicals in the field of flavoring agents.

Toxic constituents of botanicals used in food supplements

The Dutch Commodities Act Decree 'Herbal preparations' regulates the use of toxic plants and fungi in herbal preparations that are brought on the Dutch market [1]. The Decree defines herbal preparations as 'herbal substances, subjected to treatment or not, including herbal extracts, which are intended to be used by humans. In addition, 'herbal substances' are defined as 'substances that are composed of plant material'.

Plants with toxic constituents regulated by the Decree 'Herbal preparations'

The Dutch Commodities Act Decree 'Herbal preparations' defines limits to the occurrence of certain toxic constituents in herbal preparations or bans their presence altogether. The Decree includes limits for three types of phytotoxins in herbal preparations, namely toxic pyrrolizidine alkaloids, aristolochic acids and yohimbe alkaloids. The amount of toxic pyrrolizidine alkaloids in herbal preparations is limited to 1 µg/kg. Toxic pyrrolizidine alkaloids act principally on the liver where they can produce obstruction of hepatic veins resulting in veno-occlusive disease, which can be fatal. Several pyrrolizidine alkaloids are also genotoxic carcinogens. Plants known to contain pyrrolizidine alkaloids are widely used for medicinal purposes and some are even used as food (Chapter 3 of this thesis). Part I of the annex to the Decree 'Herbal preparations' lists plants that are known to contain toxic pyrrolizidine alkaloids such as comfrey (*Symphytum officinale* L.) and starflower or borage (*Borago officinalis* L.). However, the limit for toxic pyrrolizidine alkaloids extends to all plants with these constituents that are used in herbal preparations. Furthermore, the Decree forbids the presence of aristolochic acids and yohimbe alkaloids including yohimbine in herbal preparations. Aristolochic acids occur in several plants used medicinally in Traditional Chinese Medicine (TCM). Herbal preparations with *Aristolochia* spp. are carcinogenic to humans and naturally occurring mixtures of aristolochic acids are probably human carcinogens as well (Chapters 3 and 4 of this thesis).

The presence of yohimbine and related alkaloids was prohibited in 2007 following a risk assessment by the Dutch National Institute for Public Health and the Environment (in Dutch: het Rijksinstituut voor Volksgezondheid en Milieu - RIVM) on request by the VWA [4, 5]. Yohimbine is a cyclic-indole alkaloid found in the bark of the West African yohimbe tree *Pausinystalia johimbe* (K. Schum.) Pierre ex Beille. The bark contains 6% of related alkaloids, of which yohimbine is the principle alkaloid [6]. The alkaloids in this mixture are collectively called yohimbe alkaloids. Yohimbine has been used for the treatment of impotence. This alkaloid is a potent α_2-adrenoreceptor (AR) antagonist. Inhibition of α_2-ARs by yohimbine can result in an increase in blood pressure and heart rate. A wide array of clinical effects of yohimbine has been described including effects on the central nervous system (sleep disorders, anxiety, tremors), kidneys (dysuria and renal failure) and the gastrointestinal tract (anorexia, nausea and vomiting) [7, 8]. Adverse effects have been reported at daily doses of 10 to 20 mg in several studies [5, 8]. In addition to the ban of yohimbe alkaloids, the use of plant material derived from the yohimbe tree in herbal preparations was also prohibited by inserting the herb on a list of plants in part II of the annex to the Decree 'Herbal preparations'.

Part II of the annex to the Decree 'Herbal preparations' defines plants that are too toxic to be used in food or in other commodities [1]. These plants are alphabetically listed and part II of the annex is currently

comprised of 46 toxic plants and fungi such as *Aconitum napellus*, *Digitalis purpurea* and *Claviceps purpurea*. The list was derived from a now repealed Decree (het Besluit U.A.-geneesmiddelen), which defined pharmaceutical substances including botanical preparations that only could be dispensed by pharmacies [1]. The list of toxic plants in part II and the list with plants containing pyrrolizidine alkaloids defined in part I of the annex also overlap to a considerable degree with the 'CPMP list of herbal drugs with serious risks' compiled by the Committee for Proprietary Medicinal Products (CPMP) and published by the European Commission in October 1992 [9]. The Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA; at that time the acronym EMEA was used) considered the CPMP list a useful source of information on plants with intrinsic safety risks and effectively endorsed it by publishing it in its unrevised form in 2005 [10]. The Dutch repealed list of pharmaceutical substances that only could be dispensed by pharmacies and the CPMP 'list of herbal drugs with serious risks' indicate which parts of the plants are of toxicological concern. This information is however not provided for most of the banned plants that are defined in part II of the annex to the Decree 'Herbal preparations'. Table 1 shows the plants and fungi banned in part II of the annex to the Decree and presents for each of these the constituent or constituents that are responsible for, or contribute to, the toxic effects of these botanicals [6, 8, 11-17]. It also shows which parts of the plants or fungi are of toxicological concern. Where possible, plants are grouped on the basis of the presence of related toxic constituents [8].

EFSA recognized in its guidance on safety assessment of botanicals and botanical preparations that correct identification of the botanical source and botanical preparation is essential given that many species have been reclassified or renamed resulting in different scientific names for a single species. EFSA recommended therefore to follow as much as possible the nomenclature of the European Pharmacopoeia or additional sources such as Mansfeld's World Database of Agricultural and Horticultural Crops, and the Germplasm Resources Information Network (GRIN) database by the United States Department of Agriculture [3, 11, 12]. Table 1 includes in addition to the botanical names listed in part II of the annex to the Decree 'Herbal preparations', for each botanical the accepted botanical nomenclature including the author's name as identified in Mansfeld's database or the GRIN database. From Table 1 it can be derived that in several cases plant names in the list of part II of the annex have been misspelled, such as for instance *Mandragora officinarum*, which is incorrectly listed as *Mandragora officinalis*. Table 1 also includes Dutch and English common (vernacular) names. Common names may however not uniquely identify a species and are therefore not as reliable as scientific names [3]. In Traditional Chinese Medicine (TCM) use of common names is widespread and confusion over the identity of botanicals has resulted in poisonings due to the substitution of poisonous plants for innocuous species. In Belgium, 100 cases of rapidly progressing renal fibrosis were caused by substitution of *Aristolochia fangchi* for *Stephania tetrandra*. The substitution was probably caused by confusion over common names because both herbs can be identified in practice by the same traditional Chinese name, i.e. 'Fang Ji' (Chapter 4 of this thesis).

Table 1 Plants banned for use in herbal preparations listed in part II of the annex of the Dutch Commodities Act Decree 'Herbal preparations'

Category	Herbs as listed in Decree	Accepted name [b]	English name	Dutch name	Parts of concern	Toxic constituents
Cardiac glycosides	*Adonis vernalis*	*Adonis vernalis* L.	oxeye, pheasant's-eye, spring adonis	voorjaarsadonis, duivelsoog	entire plant	cardenolide glycosides: e.g. adonitoxin, cymarin
	Convallaria majalis	*Convallaria majalis* L.	lily-of-the-valley	lelietje-van-dalen	entire plant	cardenolide glycosides: e.g. convallatoxin
	Digitalis lanata	*Digitalis lanata* Ehrh.	Grecian foxglove	wollig vingerhoedskruid	entire plant	cardenolide glycosides; e.g. digoxin, digitoxin
	Digitalis purpurea	*Digitalis purpurea* L.	foxglove	vingerhoedskruid	entire plant	cardenolide glycosides: e.g. digoxin, digitoxin
	Nerium oleander	*Nerium oleander* L.	oleander, rose bay	oleander	entire plant	cardenolide glycosides: e.g. oleandrin
	Strophantus kombé	*Strophanthus kombe* Oliver	kombe	strofantus	seed	cardenolide glycosides: e.g. ouabain, K-strophanthoside
	Urginea maritima, syn. *Scilla maritima*	*Drimia maritima* (L.) Stearn	squill, sea-onion	zeeajuin	entire plant	bufadienolide glycosides: e.g. scillaren A
Tropane alkaloids	*Atropa belladonna*	*Atropa belladonna* L.	belladonna, deadly nightshade	wolfskers	entire plant	tropane alkaloids: hyoscamine, atropine
	Datura stramonium	*Datura stramonium* L.	jimsonweed, thorn-apple	doornappel	entire plant	tropane alkaloids: hyoscamine, scopolamine
	Hyoscyamus niger	*Hyoscyamus niger* L.	henbane	bilzekruid	entire plant	tropane alkaloids: hyoscyamine
	Mandragora officinalis	*Mandragora officinarum* L.	mandrake	alruin	root	tropane alkaloids: scopolamine, hyoscyamine
	Scopolia carniolica	*Scopolia carniolica* Jacq.	scopolia or Russian belladonna	klokbilzenkruid	entire plant	tropane alkaloids: hyoscyamine, scopolamine, atropine
Other alkaloids	*Aconitum napellus*	*Aconitum napellus* L.	monkshood	monnikskap	entire plant	aconitine and related aconitines
	Cephaelis acuminata, syn. *Uragoga granatensis* (including Ipecacuanhae radix [a])	*Cephaëlis acuminata* Karst. [b], *Carapichea ipecacuanha* (Brot.) L. Andersson [c]	ipecac	-	root	isoquinoline alkaloids: emetine, cephaeline

Table 1 (continued)

Category	Herbs as listed in Decree	Accepted name [b]	English name	Dutch name	Parts of concern	Toxic constituents
	Claviceps purpurea	*Claviceps purpurea* (Fr.) Tul.	ergot	moederkoorn	sclerotium	lysergic acid derivatives: ergotamine, ergometrine
	Colchicum autumnale	*Colchicum autumnale* L.	autumn crocus	herfsttijloos	entire plant	phenethylisoquinoline alkaloids: colchicine
	Genista tinctoria	*Genista tinctoria* L.	dyer's greenweed	verfbrem	aerial parts	quinolizidine alkaloids: e.g. cytisine
	Lobelia inflata	*Lobelia inflata* L.	Indian tobacco	lobeliakruid	entire plant	piperidine alkaloids: lobeline
	Pausinystalia yohimbe, syn. *Corynanthe yohimbe*	*Pausinystalia johimbe* (K. Schum.) Pierre ex Beille [c]	yohimbe	-	bark	corynane type indole alkaloids (yohimbe alkaloids): yohimbine
	Rauwolfia serpentina	*Rauvolfia serpentina* (L.) Benth. ex Kurz	snakewood	rauwolfia	entire plant	monoterpenoid indole alkaloids: reserpine
	Sarothamnus scoparius, syn. *Cystisus scoparius*	*Sarothamnus scoparius* (L.) Wimmer ex Koch	broom	bremkruid	aerial parts	quinolizidine alkaloids: sparteine, lupanine
	Solanum dulcamara	*Solanum dulcamara* L.	bittersweet	bitterzoet	entire plant	glycoalkaloids: e.g. solanine
	Strychnos nux-vomica	*Strychnos nux-vomica* L.	nux-vomica	braaknootboom	seed	monoterpenoid indole alkaloids: strychnine, brucine
	Vinca minor	*Vinca minor* L.	common periwinkle	kleine maagdepalm	plant	indole alkaloids: e.g. vincamine
Possible genotoxic carcinogens	*Croton tiglium*	*Croton tiglium* L.	purging croton	-	oil, seed	tetracyclic diterpenoid alcohol phorbol and esters
	Juglans regia, with the exception of the nuts	*Juglans regia* L.	walnut	walnoot	shell	naphthoquinone: juglone
	Rubia tinctorum	*Rubia tinctorum* L.	madder	meekrap	root	anthraquinones: e.g. lucidin
Others	*Artemisia cina*	*Seriphidium mogoltavicum* (Polj.) Y.R. Ling [b]; *Artemisia cina* O. Berg [c]	Levant wormseed	echt wormkruid	unopened flower-buds	sesquiterpenoid lactones: santonin

Table 1 (continued)

Category	Herbs as listed in Decree	Accepted name [b]	English name	Dutch name	parts of concern	toxic constituents
	Artemisia maritima	*Seriphidium maritimum* complex [b], *Artemisia maritima* L. [c]	sea wormwood	zeealsem	flower-buds	sesquiterpenoid lactones: e.g. santonin
	Brassica nigra, with the exception of the use of the seed in foods	*Brassica nigra* (L.) Koch	black mustard	zwarte mosterd	seed	isothiocynate glycoside: sinigrin
	Bryonia alba	*Bryonia alba* L.	white bryony	heggerank	root	cucurbitacines
	Chenopodium ambrosioides (var. *anthelminthicum*)	*Chenopodium ambrosioides* var. *anthelminticum* (L.) A. Gray [b]; *Dysphania anthelmintica* (L.) Mosyakin & Clemants [c]	wormseed	welriekende ganzevoet	essential oil	monoterpenoids: ascaridol
	Chrysanthemum vulgare, syn. *Tanacetum vulgare*	*Tanacetum vulgare* L.	common tansy	boerenwormkruid	flowering tops, leaves	bicyclic monoterpenoid: thujone
	Citrullus colocynthidis	*Citrullus colocynthis* (L.) Schrad.	bitter apple	kolokwint, kwintappel	entire plant	cucurbitacines
	Convolvulus scammonia	*Convolvulus scammonia* L.	scammony	-	resin	jalapin
	Dryopteris filix-mas	*Dryopteris filix-mas* (L.) Schott	male fern	mannetjesvaren	rhizome	phloroglucinol derivatives
	Exogonium purga, syn. *Ipomoea purga*	*Ipomoea purga* (Wender.) Hayne	jalap	jalappe	tuber, resin	convolvulin, jalapin
	Juniperus sabina	*Juniperus sabina* L.	savin	zevenboom	essential oil; leaves, twigs	sabinene, thujone
	Ledum palustre	*Rhododendron tomentosum* Harmaja [c]	-	moerasrozemarijn	entire plant	terpenes, e.g. ledol

Table 1 (continued)

Category	Herbs as listed in Decree	Accepted name [b]	English name	Dutch name	Parts of concern	Toxic constituents
	Lycopus europaeus	*Lycopus europaeus* L. [c]	gypsywort	wolfspoot	unspecified	uncertain
	Mallotus philipinensis, syn. *Rottlera tinctoria*	*Mallotus philippinensis* (Lam.) Müll. Arg.	kamala tree	kamala	fruit	phloroglucinol derivatives: e.g. rottlerin
	Piper methysticum	*Piper methysticum* G. Forst.	kava	kava kava	root	kavalactones
	Podophyllum peltatum, with the exception of the fruit	*Podophyllum peltatum* L.	mayapple	voetblad, meiappel, eendvoet	root, resin	podophyllotoxin
	Pulsatilla vulgaris, syn. *Anemona pulsatilla*	*Anemone pulsatilla* L. [c]	pasqueflower	wildemanskruid, paarse anemoon	entire plant	protoanemonin
	Ricinus communis	*Ricinus communis* L.	castor-bean	kruisboom, wonderboom	seed, oil	ricin
	Teucrium chamaedrys	*Teucrium chamaedrys* L.	wall germander	gamander, wilde salie	flowering plant	furano neo-clerodana diterpenoids: e.g. teucrin A

[a] Radix Ipecacuanhae consists of the dried roots and rhizomes of *Cephaelis ipecacuanha* (Brot.) A. Rich., or of *C. acuminata* (Benth.) Karst., or of a mixture of both species [13]

[b] According to Mansfeld's World Database of Agricultural and Horticultural Crops [11]

[c] According to GRIN database by the United States Department of Agriculture [12]

References: [6, 8, 11-17]

RIVM reviewed the toxicity of the plants and fungi currently defined in part II of the annex to the Decree and concluded that only for one herb (*Convolvulus scammonia*) on the list no evidence could be found supportive of maintaining its position on the list [8]. The resin of this botanical is however listed in the 'CPMP list of herbal drugs with serious risks' as a drastic laxative with irritant properties [9]. The herb has been included in lists of banned plants in other Member States too and is listed in the EFSA Compendium of botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern [14]. The list of plants banned for use in herbal preparations established in part II of the annex to the Decree 'Herbal preparations' has been amended several times. To date, the herbs *Magnolia officinalis*, *Stephania tetrandra* and *Lycium barbarum* have been removed from the initial list by the Dutch Ministry of Health because of a risk assessment, which showed that these herbs were not toxic [18]. The risk assessment was conducted by RIVM on request by the VWA. While *Stephania tetrandra* in itself is not toxic, the potential for substitution with *Aristolochia* species poses a potential health risk and both VWA and RIVM advised therefore to maintain the ban on this botanical [19, 20].

New additions to the list of banned herbs were kava kava (*Piper methysticum* G. Forst.) in 2003 and, already described, yohimbe in 2007. Kava kava was included on the list because of cases of liver toxicity reported in several countries. Risk assessments by governmental bodies from Germany and the UK were central to discussions whether kava kava should be removed from the Dutch market [21]. The German Federal Institute for Drugs and Medical Devices - Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM) in Bonn collected 39 spontaneous reports where kava kava was linked to poor liver function, liver infections, necrosis and in three cases to fatal effects. In total 18 spontaneously reported cases were sufficiently documented in order to be assessed for causality. Based on these 18 cases and an additional 2 German cases reported in literature it was concluded that kava kava possesses the potential to cause severe liver toxicity showing a distinctive pattern of effects peaking at 3 to 4 months after the start of medication. In addition, the effects were likely to be more severe at higher doses [22]. BfArM repealed authorizations for kava kava products to treat nervous states such as anxiety, tension and restlessness in June 2002.

In the UK, an expert group was established by the Medicines and Healthcare products Regulatory Agency (MHRA) to assess the safety of kava kava. The expert group determined in 2002 after reviewing 68 cases using a causality assessment method according to WHO criteria that this botanical was associated with an unacceptable risk of idiosyncratic hepatotoxicity. Kava kava products were subsequently removed from the UK market early 2003. All available data including details of an additional 42 cases were again reviewed in October 2005. On a total of 110 received case reports, nine cases had a fatal outcome and in an additional nine cases patients received liver transplants and survived. The expert group concluded that the prohibition of kava kava remained justified and proportional. It was decided that none of other proposed risk minimization measures including label warnings would allow the safe use of kava kava [23].

It can be concluded that in the Netherlands the Decree 'Herbal preparations' includes limits for three types of phytotoxins in herbal preparations and prohibits the use in herbal preparations of 46 plants and fungi that are too toxic to be used in food or in other commodities. The constituents responsible for the toxic effects of these

46 plants are also found in plants not specifically regulated by the Decree. These plants not regulated by the Decree but containing identical or related toxic constituents are discussed in the next section.

Plants not regulated by the Decree 'Herbal preparations' but with identical or related toxic constituents

Where the Dutch list of banned botanicals contains only individual species, the 'CPMP list of herbal drugs with serious risks' also defines several genera in which all species possibly contain identical or related toxic constituents; examples are the genera *Aconitum* and *Bryonia*. The only *Aconitum* species banned in the Netherlands for use in herbal preparations is *A. napellus*, but botanical materials from other *Aconitum* species are also available as herbal preparations on the Dutch market. The roots of the species *A. carmichaeli* (Chuan Wu) and *A. kusnezoffii* (Cao Wu) are used in TCM for the treatment of musculoskeletal disorders and these species contain the potent neurotoxin and cardiotoxin aconitine, as does *A. napellus* (Table 1). Traditional Chinese herbal preparations are sold in the Netherlands in specialized shops where often therapists practice TCM as well. In Hong Kong several cases of herb-induced aconitine poisonings are treated in hospitals almost every year [24]. Certain preparation techniques are used in TCM in order to reduce aconitine and other toxic aconitine type alkaloids in *Aconitum* plant material, but levels of these aconitines can differ significantly between preparations [25]. Because many *Aconitum* species have been described, it is likely that several more of these species contain aconitines and that use of herbal preparations based on these plants can result in adverse effects. RIVM suggested to consider imposing a limit for, or a ban on the presence of aconitine and related aconitines in herbal preparations, analogous to the ban on aristolochic acids in herbal preparations already in place in the Decree [8]. A ban on the presence of aconitine and related aconitines in herbal preparations would be, in effect, in agreement with the CPMP position that all parts of all *Aconitum* spp. are herbal drugs with serious risks without any accepted benefit [26].

In the review of all plants from part II of the annex to the Decree "Herbal preparations', RIVM suggested to follow a similar strategy for several other categories of toxic constituents that could be found in plants included in the Dutch list of herbs that are banned from being used in herbal preparations (Table 2) [8]. Exposure to these constituents at typical dose levels can result in severe toxic effects. RIVM suggested that other botanicals with these constituents can also be cause for concern and that these botanicals were likely candidates for risk assessments. For example, strychnine is also found in other *Strychnos* species than *S. nux-vomica*, which is the only *Strychnos* species currently banned in the Netherlands, and these other toxic *Strychnos* species are used in Asia and Africa for several complaints [14, 27]. However, other categories of highly toxic constituents of botanicals exist and the toxic constituents presented in Table 2 cover only a certain part of the botanicals on the Dutch market for which possible health concerns exist. For a more extended overview, the reader is referred to the Compendium published by EFSA [14].

Table 2 Categories [(a)] of constituents of banned herbal preparations in part II of the annex to the Commodities Act Decree 'Herbal preparations' that can result in severe toxic effects

Constituents	Examples of herbs with constituent
Cardiac (cardenolide) glycosides	*Adonis vernalis* L., *Convallaria majalis* L., *Digitalis lanata* Ehrh.
Tropane alkaloids (hyoscamine, atropine or scopolamine)	*Atropa belladonna* L., *Datura stramonium* L.
Thujone	*Tanacetum vulgare* L., *Artemisia absinthium* L.
Genotoxic and carcinogenic constituents	*Juglans regia* L.
Aconitine and closely related aconitines	*Aconitum napellus* L.
Colchicine	*Colchicum autumnale* L.
Ergotamine, ergometrine and other ergot alkaloids	*Claviceps purpurea* (Fr.) Tul.
Yohimbe alkaloids	*Pausinystalia johimbe* (K. Schum.) Pierre ex Beille
Podophyllotoxin	*Podophyllum peltatum* L.
Protoanemonin	*Anemone pulsatilla* L
Ricin	*Ricinus communis* L.
Sparteine	*Sarothamnus scoparius* (L.) Wimmer ex Koch
Strychnine	*Strychnos nux-vomica* L.

[a)] As indicated by the Dutch National Institute for Public Health and the Environment (RIVM) [8]

It can be concluded that there are several plants not regulated by the Dutch Decree 'Herbal preparations' but containing identical or related toxic constituents, which also give reason for concern. The current regulation and legislation in the Netherlands and Europe needs to be updated to address these possible health risks. This also holds for another class of botanicals, which are used in herbal preparations and not specifically regulated, but flagged by risk assessors because of health concerns. Examples of this class of botanicals are presented in the next section.

Botanicals used in food supplements not specifically regulated but flagged by risk assessors due to health concerns

In 2006, the HMPC of EMA (then known as the EMEA) published a statement on a potential connection between *Cimicifugae racemosae* rhizoma (black cohosh root) and cases of hepatotoxicity. EMA advised patients to stop taking the root of black cohosh (*Cimicifuga racemosa* (L.) Nutt., which is the synonym of *Actaea racemosa* L. [12]), upon signs and symptoms suggestive of liver injury and subsequently consult their doctor. Healthcare professionals were also asked to report suspected hepatic reactions [26]. In 2007, the HMPC published an update of the assessment of the case reports connected to black cohosh root use [28]. Overall, all discussed cases of literature and pharmacovigilance reports were poorly documented. Of the 18 cases that could be scored using a standardized causality assessment method (RUCAM), three cases could be classified as possible and two as probable. Since then discussions on the hepatotoxicity of black cohosh have been ongoing. One group reviewed 69, in general poorly documented, cases of which only one involved a possible causal relationship with

liver disease resulting in the conclusion that these cases did not, or only to a limited extent, support the hepatotoxicity of black cohosh [29]. Another group contested this conclusion and proposed that a label caution for black cohosh products should be made mandatory following similar proposals by British, Canadian and Australian governmental agencies due to growing evidence supporting the hepatotoxic potential of this botanical [30]. In Australia, medicines containing *Cimicifuga racemosa* are required since 2008 to list the following warning: 'In very rare cases, Black cohosh has been associated with liver failure' [31].

Another herb linked to liver toxicity is greater celandine (*Chelidonium majus* L.). Greater celandine contains at least 20 alkaloids including chelidonine, protopine and berberine, which belong in total to three groups of alkaloids. The herb is included in the EFSA Compendium and the benzophenantridine alkaloids chelidonine and others are listed as the chemicals of concern. Several Member States banned the sale of this botanical as food [14]. An ethanolic greater celandine extract was orally administered for 2 or 4 weeks to 48 Wistar rats in doses corresponding to 1.5 and 3 g/kg bw/day of herbal drug, equivalent to 5.5 and 11 mg/kg bw/day of total alkaloids. According to the authors, the botanical drug did not alter hepatic function but GSH levels and SOD activity were significantly reduced after 4 weeks of treatment. It was advised that long-term use should be avoided [32]. In Germany, BfArM limited in 2005 the intake of alkaloids from greater celandine to 2.5 µg per day based on an *in vitro* assay with rat hepatocytes. In 2008, this limit was expanded to 2.5 mg of total alkaloids per day (equivalent to 0.04 mg/kg bw/day for a 60 kg person) based on a NOAEL of > 3.69 mg/kg bw/day obtained in a 6 month toxicity study in rats [33]. BfArM also assessed 48 cases of liver toxicity linked to herbal medicinal products containing greater celandine. In total, for 17 cases the causality was rated as probable, for 26 cases as possible and for one case as absent. Another 4 cases could not be assessed [33, 34]. One case report of toxic liver failure had a fatal outcome and the causality with greater celandine use was assessed as possible [34]. In the Netherlands, several botanical food supplements are on the market that contain greater celandine.

In the 'CPMP list of herbal drugs with serious risks', *Angelica archangelica* L. is listed because of the presence of phototoxic furanocoumarins (or furocoumarins) [9]. A review of the available data by the HMPC of EMA showed that several furocoumarins, such as 8-methoxypsoralen, combined with UV-light pose a genotoxic and carcinogenic hazard to humans. 8-Methoxypsoralen is found in *Angelica archangelica* L. and in the fruits of *Ammi majus* L. This substance is used, followed by UVA light exposure, to treat vitiligo or psoriasis [6, 35]. Intakes of furocoumarins through botanical herbal medicinal products or herbal preparations (food supplements) will add to an already considerable exposure from various foods, such as celery. HMPC concluded that the health risk to humans associated with the exposure to furocoumarins from herbal preparations should be assessed [35]. Interestingly, the plant is not included in the EFSA Compendium but two other *Angelica* species are due to the presence of furocoumarins [14].

In 2006, following a rapid alert, RIVM assessed on request by the VWA the risks presented by cyanogenic glycosides in bitter almonds (*Prunus dulcis* (Mill.) D.A. Webb var. *amara* (DC.) Buchheim) and kernels of apricots (*P. armeniaca* L.). In the United States, apricot kernels, also referred to as apricot pits, are sold in health food stores and are believed by some to act as anticancer agents, although efficacy has not been demonstrated [6]. Apricot kernels and bitter almonds are sold in the Netherlands in food retail shops, health food

stores and via Internet as well. RIVM concluded that a dose of 0.6 mg cyanide/kg bw/day could possibly be fatal for adults and that 30 g of kernels could represent a lethal dose, assuming that the cyanogenic glycosides present in the kernels could release 1000 mg of cyanide/kg kernels. However, cyanide levels of up to 3000 mg/kg kernels have been reported [36]. At these higher concentrations, lower doses than 30 g of kernels could also be lethal. RIVM was not able to derive an acute oral reference dose due to the limited availability of data on the acute toxicity of cyanide via the oral route [37].

Botanicals with laxative properties are relatively popular. A number of glycosides with aglycones related to anthracene are present in botanicals such as cascara sagrada (*Frangula purshiana* (DC.) J. G. Cooper), frangula (*Frangula alnus* Mill.), aloe (*Aloe vera* (L.) Burm. f. (syn. *A. barbadensis* Mill.); *Aloe ferox* Mill.), rhubarb (*Rheum palmatum* L.) and senna (*Senna alexandrina* Mill.) [6, 12]. All of these plants are listed in the EFSA Compendium because of health concerns raised by the presence of anthracene derivatives. In addition, warning labels are mandatory for several of these botanicals in Belgium [14]. The anthracene derivatives found in these plants include anthraquinones, anthrones, dianthrones and related compounds that are mostly present as glycosides [6, 38]. These anthracene derivatives are also called anthranoids [38]. Anthranoid compounds are stimulant cathartics and exert their action by increasing the tone of the smooth muscle in the wall of the colon and stimulate the secretion of water and electrolytes into the large intestine [6].

Anthranoid containing herbal drugs are recommended for short-term treatment (one to two weeks) of constipation [39]. Long-term use of herbal preparations with anthranoid compounds may however lead to watery diarrhea resulting in excess loss of electrolytes and water. Stimulant laxatives are habit-forming and long-term use may result in laxative dependence and loss of normal bowel function [6]. Abnormalities such as melanosis coli, enteric changes and neoplastic changes have been linked to anthranoid compounds [39, 40]. The association between colorectal neoplasm in humans and the use of herbs with these compounds remains however inconclusive [40]. HMPC assessed cascara, frangula bark, aloe, rhubarb and senna (pods and leaves) and concluded that use of herbal medicinal products based on these botanicals for more than one to two weeks requires medical supervision. HMPC also warned against impaired function of the intestine and dependence on these laxatives linked to long-term use and defined other warnings and precautions for use [41-46]. It can be concluded that it would be prudent not to use these stimulant laxatives for prolonged periods without medical supervision.

Use of plants with flavoring properties in conventional foods and herbal preparations

In addition to the use of botanicals to improve health or treat disease, many plants are used traditionally to enhance the flavor or aroma of foods. The use of plants for flavorings and food ingredients with flavoring properties in foods is regulated by European food legislation, which is the basis of provisions for these products in the Dutch Commodities Act. Recently, the European Union updated the current legislation on flavorings (Directive 88/388/EEC) resulting in Regulation No 1334/2008, which shall apply from 20 January 2011 [47, 48]. Flavoring substances are defined chemical substances, including natural flavoring substances. The Committee of Experts on Flavouring Substances of the Council of Europe concluded that a number of substances occurring naturally in

botanical source materials for flavorings and food ingredients with flavoring properties, raise toxicological concern. In those cases where the Scientific Committee on Food (SCF) - or EFSA - confirmed that the substance in question raised toxicological concern, the substance was regarded as an undesirable substance and consequently prohibited by Regulation No 1334/2008 to be added to food in the form of the pure, chemically defined substance. These undesirable substances can also be present in flavoring preparations and food ingredients with flavoring properties due to their natural occurrence in plants that are used as source materials for these flavoring preparations and ingredients. Maximum levels for these undesirable substances have therefore been established for foods that contribute most to the human intake of these substances [48]. Table 3 presents these substances that in effect are toxic principles of which the addition to foods will be prohibited by Regulation No 1334/2008. Also shown in Table 3 are examples of botanicals that contain these toxic principles as well as the most important toxic effects of these substances [49-55]. The effects of several of these compounds are discussed in more detail in Chapter 3 of this thesis.

Currently, Directive 88/388/EEC still applies and this directive also includes a list of compounds that are banned from being added to food in a pure, chemically defined form. The list in Directive 88/388/EEC is composed of substances that, except for santonin and isosafrole, are also prohibited by Regulation No 1334/2008 from being used in food in a pure form [48]. Santonin is found in the plants *Artemisia cina* and *A. maritima*, which are banned for use in herbal preparations in the Netherlands (Table 1). Isosafrole is a weak rodent hepatocarcinogen. It occurs sporadically and then only together with safrole. SCF concluded that any measure to restrict exposure to safrole in food would cover isosafrole as well [56]. As well as the substances banned by Directive 88/388/EEC (with the exception of santonin and isosafrole), Regulation No 1334/2008 additionally prohibits the use of capsaicin, estragole, menthofuran, methyleugenol and teucrin A as pure substances in food [47, 48].

One of the substances banned both by Directive 88/388/EEC and Regulation No 1334/2008 from being added in its pure form to food is the carcinogen beta-asarone [47, 48]. In addition to beta-asarone, its isomer alpha-asarone is also a mammalian carcinogen, but the addition of alpha-asarone to food as a pure substance is not prohibited. It is a constituent of several botanicals such as *Asarum europaeum* L., *Asarum arifolium* Michx and *Daucus carota* L. Asarones have also been reported to occur in *Sassafras albidum* (Nutt.) Nees and *Acorus gramineus* Ait. The HMPC of EMA advised that the concentration of both asarones in herbal medicinal products should be reduced to a minimum. Following the setting of maximum levels for beta-asarone in European food legislation, HMPC proposed a provisional limit of exposure from herbal medicinal products of 115 µg/day, equivalent to 2 µg/kg bw/day for a 60 kg person [57].

Table 3 Substances effectively prohibited from 20 January 2011 by Regulation No 1334/2008 (annex III) from being added as such to food [48]

Toxic substance	Toxic action/ effect	Examples of herbs with constituent
Agaric acid	Highly irritating effect on intestinal mucosa	Dried white agaric mushroom (*Fomes officinalis* (Vill:Fr) Ames)
Aloin	Laxative	*Aloe vera* (L.) Burm. F., (= *A. barbadensis*), *Aloe ferox* Mill.
Beta-asarone	Carcinogenic in rodents, suspected genotoxic carcinogen	Rhizome of calamus (*Acorus calamus* L.)
Capsaicin	Local irritant, increased risk for gastric cancer observed at doses above 200 mg per day	Fruits of peppers (*Capsicum annuum* L. and *C. frutescens* L.)
Coumarin (1,2-benzopyrone)	Hepatotoxicity	Cinnamon (*Cinnamomum verum* J. Presl) and tonka beans (*Dipteryx odorata* (Aubl.) Willd.)
Estragole (1-allyl-4-methoxybenzene)	Genotoxic carcinogen	Sweet basil (*Ocimum basilicum* L.) and tarragon (*Artemisia dracunculus* L.)
Hydrocyanic acid	Acute cyanide poisoning, chronic effects on nervous system and thyroid	Present as cyanogenic glycosides at least in 2000 plants, including bitter almonds and apricots (*Prunus dulcis* (Mill.) D.A. Webb var. *amara* (DC.) Buchheim, *P. armeniaca* L., respectively)
Hypericine	Enhanced photosensitivity	St. John's wort (*Hypericum perforatum* L.)
Menthofuran	Hepatotoxicity, nephrotoxicity	Peppermint oil (*Mentha* x *piperita* L. var. *piperita*) and pennyroyal oil (*Mentha pulegium* L.), buchu leaf oil (*Agathosma betulina* (P. J. Bergius) Pillans)
Methyleugenol (4-allyl-1,2-dimethoxybenzene)	Genotoxic carcinogen	Nutmeg (*Myristica fragans* Houtt.), tarragon, basil, star anise (*Illicium verum* Hook. F.)
Pulegone	Hepatotoxicity, nephrotoxicity	Peppermint oil and pennyroyal oil
Quassin	Possible reproductive toxicity	Quassia (*Quassia amara* L. or *Picrasma excelsa* (Sw.) Planch)
Safrole (1-allyl-3,4-methylene dioxy benzene)	Genotoxic carcinogen	Nutmeg and mace (*Myristica fragans* Houtt.), cinnamon, black pepper (*Piper nigrum* L.) and sweet basil
Teucrin A	Hepatotoxicity	Germander (*Teucrium chamaedrys* L.)
Thujone (alpha and beta)	Neurotoxicity	Sage (*Salvia officinalis* L.) and several *Artemisia* species such as wormwood (*A. absinthium* L.)

References: [49-55]

Furthermore, HMPC reviewed the SCF opinion on pulegone and menthofuran (Table 3) in relation to herbal medicinal products containing peppermint oil (*Mentha piperita* L.), mint oil (*M. canadensis* L., syn. *M. arvensis* var *piperascens* Malinv. Ex Holmes) and pennyroyal oil (*M. pulegium* L. or *Hedeoma pulegoides* (L.)

Pers) [58, 59]. Following the SCF, HMPC also concluded that pulegone is a hepatotoxin. Daily pulegone doses exceeding the TDI for food are commonly encountered in herbal medicinal products in Europe but no certain cases of liver toxicity in humans have been reported. HMPC concluded that the use of pennyroyal oil in unlicensed products should be discouraged [58]. HMPC also considered another toxic substance from Table 3, estragole, which is also found in a wide range of aromatic plants. It was concluded that estragole is a naturally occurring genotoxic carcinogen, but that it does not represent a significant cancer risk to users of herbal medicinal products given the low levels of exposure resulting from consumption of these products (short time use in adults at recommended posology) [60]. HMPC derived similar conclusions for methyleugenol, another alkenylbenzene included in Table 3 [61].

The use of the Margin of exposure (MOE) approach developed by EFSA [62] has been advocated for the risk assessment of constituents of botanicals which are genotoxic and carcinogenic [63]. The MOE can be obtained by dividing the Benchmark Dose Lower Confidence Limit associated with an extra 10% cancer risk relative to background incidence (BMDL10) by the estimated dietary intake of a compound. EFSA proposed that MOEs of 10,000 or higher - taking into account certain criteria - would be of low concern from a public health point of view and might be considered as a low priority for risk management actions [62]. For example, for the intake of methyleugenol from spices MOEs of 15,400-158,000 for females and MOEs of 4,400-64,000 for males were calculated indicating that the use of these spices should be considered as a low priority for risk management [63]. However, for specific botanicals, MOEs might prove to be lower than 10,000 as was shown for estragole from infusions of bitter fennel fruits as tea for which MOEs in the range of 34 to 1000 could be calculated [64].

Glycyrrhizinic acid is a triterpenoid saponin compound extracted from the roots and rhizomes of the liquorice plant (*Glycyrrhiza glabra* L.) that is used for its sweetness in a range of foodstuffs such as liquorice confectionery. This active principle can cause water and sodium retention and increased excretion of potassium, which over a prolonged period may result in hypokalaemia, hypernatraemia, edema, hypertension and cardiac disorders (Chapter 3 of this thesis). Both EFSA and SCF evaluated the safety of this constituent and its ammonium salt and considered it prudent that regular ingestion should not exceed 100 mg/day [65, 66]. Directive 2008/5/EC defines compulsory indications on confectionery and beverages in case these foods contain glycyrrhizinic acid above certain levels. At relatively low levels it is sufficient to include ‘contains liquorice’ into the labeling but at higher levels the following statement is to be included: ‘contains liquorice – people suffering from hypertension should avoid excessive consumption [67]. This obligation has been transposed to the Dutch Commodities Act Decree ‘Labeling of foods’. The rule does not apply to botanical food supplements with *Glycyrrhiza glabra*, however.

Contaminants of botanical food supplements

Botanical food supplements can become adulterated with other botanicals than the intended botanical ingredients. In 1997, the US Food and Drug Administration (FDA) investigated a case of a patient presenting with a toxic serum digoxin level after using a botanical preparation. It was shown that an ingredient of the preparation listed as ‘plantain’ was adulterated by *Digitalis lanata* Ehrh., which contains cardiac glycosides. Plantain (not to be

confused with the fruit of the banana plant of the genus *Musa*) is an herb of the genus *Plantago*. The potentially contaminated plantain powder had been distributed to at least 150 manufacturers, distributors, and retailers [68]. If this botanical would be found as an adulterant of herbal preparations on the Dutch market, specific provisions would already be in place to enforce food safety because *Digitalis lanata* Ehrh. is banned for use in herbal preparations in the Netherlands (Table 1).

In the next example herbal preparations were found on the Dutch market that were adulterated by a botanical that was not specifically banned from use in food. In 2001, a series of reports were received by the VWA of adverse effects linked to a herbal tea mixture with star anise and other herbs. Investigation by the VWA showed that the tea contained fruits of an unknown star anise species lacking the anise flavor that is characteristic for Chinese star anise (*Illicium verum* Hook. f.), which is the star anise species used in food. The unidentified species was shown to contain the neurotoxin anisatine, which can cause epileptic seizures [69]. The absence of specific legal provisions seriously complicated enforcement actions. Measures to protect consumers were therefore taken by the VWA on the basis of the ban of placing foods on the market that are injurious to health or unfit for human consumption. Furthermore, it was interesting to note that VWA inspectors were informed that there existed at that time a shortage of Chinese star anise and that as a result star anise of inferior quality was used in food, which normally would not have been used for this purpose. It is likely that the toxic star anise fruits found in the tea were collected in the wild. This case illustrates that, as EFSA recognized in 2004, misidentification of plants harvested from the wild is an important problem regarding the safety of herbal preparations [70].

Contamination of herbal preparations with a wide range of other substances than toxic plant material has been described. Contaminants included heavy metals, synthetic drugs and other undesirable substances [70, 71]. Botanical food supplements are frequently adulterated with synthetic drugs such as sibutramine or illegal analogs of sildenafil, which is the active substance of Viagra®. Synthetic drugs are also found relatively often in botanical food supplements sampled by the VWA and other Dutch governmental services. These adulterations are of major toxicological concern and the related health risks have increased in the last decade [71-73]. The presence of synthetic drugs in food supplements can result in the classification of the product as medicinal product and this topic will be discussed further in the next section of this chapter, which focuses on borderline products.

Recent reports of high levels of lead, cadmium and mercury in certain food supplements suggest that use of these products can contribute significantly to human exposure to these metals. The European Commission introduced therefore maximum levels for these metals in food supplements in Regulation (EC) No 1881/2006, which apply since July 2009 [74, 75]. No limit has yet been proposed for arsenic. In the Netherlands, Regulation (EC) No 1881/2006 is linked to the Dutch Commodities Act, but its provisions directly apply to foods on the Dutch market. In Chapter 5 the presence of lead, mercury and arsenic is investigated in herbal preparations used in Asian traditional medicine systems, which were sampled on the Dutch market.

Mycotoxins are another important group of contaminants that can be found in botanical food supplements. These mycotoxins are in most cases produced by fungi that contaminated the botanical material. Regulation (EC) No 1881/2006 sets maximum levels for certain contaminants in foodstuffs including a limit for the

mycotoxin ochratoxin A on liquorice root used as an ingredient of herbal infusions [74, 76]. In some cases mycotoxins are the product of a fungal ingredient of a herbal preparation such as for instance the fungus *Monascus purpureus* that is used to produce red yeast rice. This herbal preparation is used for healthy blood lipid levels. An important active constituent of red yeast rice is monacolin K (the effects of this constituent will be discussed in the next section on borderline products) [77]. Moreover, the fungus *Monascus purpureus* also produces a secondary metabolite called citrinin. This hepato-nephrotoxin was found in each of 12 commercial 'Monascus red mould rice' products that originated from China and were obtained from several European companies [78].

Regulation (EC) No 1881/2006 also defines limits for benzo[a]pyrene in several foods, but not for food supplements. Benzo[a]pyrene is a polycyclic aromatic hydrocarbon (PAH) and several of these, including benzo[a]pyrene, are genotoxic and carcinogenic. The Dutch Commodities Act includes since January 2006 a maximum level of 10 µg/kg benzo[a]pyrene for food supplements with botanical ingredients and a maximum of 2 µg/kg benzo[a]pyrene for supplements without these ingredients [79]. Chapter 7 of this thesis presents data obtained on the occurrence of genotoxic and carcinogenic PAH in different food supplement categories with special emphasis on botanical food supplements.

Borderline products

Certain borderline cases exist regarding the classification of products with substances, notably herbal extracts, that are used both in food supplements and for manufacturing proprietary medicinal products, in particular traditional herbal medicinal products [80]. The classification of products as medicinal products or as food products is an important matter that can significantly influence the availability of a product on the market. For instance, there used to be several herbal preparations on the Dutch market that contained ephedrine. These alkaloids are found in several species of the genus *Ephedra*. Herbal preparations regulated by food law with ephedrine alkaloids were predominantly sold as weight-loss supplements or as 'herbal ecstacy'. In 2004, the Dutch Minister of Health concluded however that because of concerns regarding the toxicity of ephedrine alkaloids (see Chapter 3 of this thesis), products with these substances could only be allowed on the market as medicinal products for which a market approval is mandatory [81]. Because the registration procedure for medicinal products is costly, none of the food supplements with ephedrine alkaloids are now being sold as medicinal products. Other botanical products with stimulant activity have been brought on the market as an alternative to products with *Ephedra* spp. that contain synephrine as an active substance, which is chemically related to ephedrine (Chapter 3 of this thesis). This compound can be found in significant quantities in the peel of the immature fruit of the bitter orange (*Citrus aurantium* L. ssp. *aurantium* L.) but also in the whole mature fruit. Extracts used in many food supplements contain synephrine levels that are often much higher than the synephrine concentrations reported for traditional extracts of the dried fruit or peel [64]. It has been debated whether synephrine is a medicinal product or a food due to its similarities to ephedrine alkaloids in activity and toxicity. No official position has been issued by the Dutch competent authorities yet.

Discussions on the legal status of another botanical, red yeast rice, culminated in influential case law by the European Court of Justice which further refined the criteria for the classification of products as medicinal products (case C-140/07 'Hecht-Pharma GmbH' of January 2009) [82]. Red yeast rice contains monacolin K, which is synonymous with lovastatin, an inhibitor of cholesterol synthesis contained in a number of prescription medicinal products. The recommended dose of the product under consideration in the court case amounted to a daily consumption of 1.33 to 4 mg of monacolin K, which is low in comparison with the daily consumption of 10 to 80 mg recommended for lovastatin [82]. Red yeast rice is steamed rice fermented by the fungus *Monascus purpureus* [77, 78]. Adverse effects such as hepatotoxicity and myopathy have been linked to red yeast rice and monacolin K and other monacolins present in this preparation [77, 83]. From the judgment of the European Court of Justice in case C-140/07 it can be concluded that for a product to be classified as a medicinal product on the basis of its pharmacological effect, it must be able to significantly affect physiological functions in human beings. And when a classification as medicinal product is considered for a (botanical) product it is up to the competent authority to prove that this particular product appreciably affects physiological functions by conducting an scientific assessment of the specific pharmacological, immunological or metabolic properties taking into account its composition – including its content in active substances – and intended use levels.

Conclusions

In the Netherlands, the use of botanical ingredients in food supplements is covered by national legislation: the Commodities Act Decree 'Herbal preparations'. In absence of European legislation for this category of products it is pertinent to keep the Decree up to date in order to deal with emerging health risks posed by changes in the market for food supplements with botanical ingredients. Recent work by RIVM, EMA and EFSA has shown that in addition to the three toxic herbal constituents (toxic pyrrolizidine alkaloids, yohimbe alkaloids and aristolochic acids) defined in the Decree, other groups of substances found in several food supplements with botanical ingredients are also of present concern. EFSA developed a new methodology for the safety assessment of botanicals used in food supplements and published a Compendium of botanicals that raise toxicological concern to be used as input for risk assessments. The tools developed by EFSA can then be used to assess the risks of botanicals not yet specifically covered by European and Dutch food safety legislation. European and Dutch risk assessment bodies concluded that several botanicals not specifically regulated by the Decree pose health risks. Opinions of these risk assessment bodies should be considered when a review of Decree 'Herbal preparations' is conducted. In several of these risk assessments label warnings are advocated for specific botanicals. It should be discussed whether label warnings are desired on food commodities including herbal preparations because foods are not expected by consumers to cause adverse effects. Altogether, it is concluded that the current regulation and legislation in the Netherlands and Europe needs to be updated to address health risks relating to botanicals and botanical preparations that are identified to be of concern.

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CHAPTER
3

Based on:

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Wim Spiegelenberg and Gerrit M Alink

Molecular mechanisms of toxicity of important food-borne phytotoxins

Molecular Nutrition & Food Research 2005, 49, 131-158.

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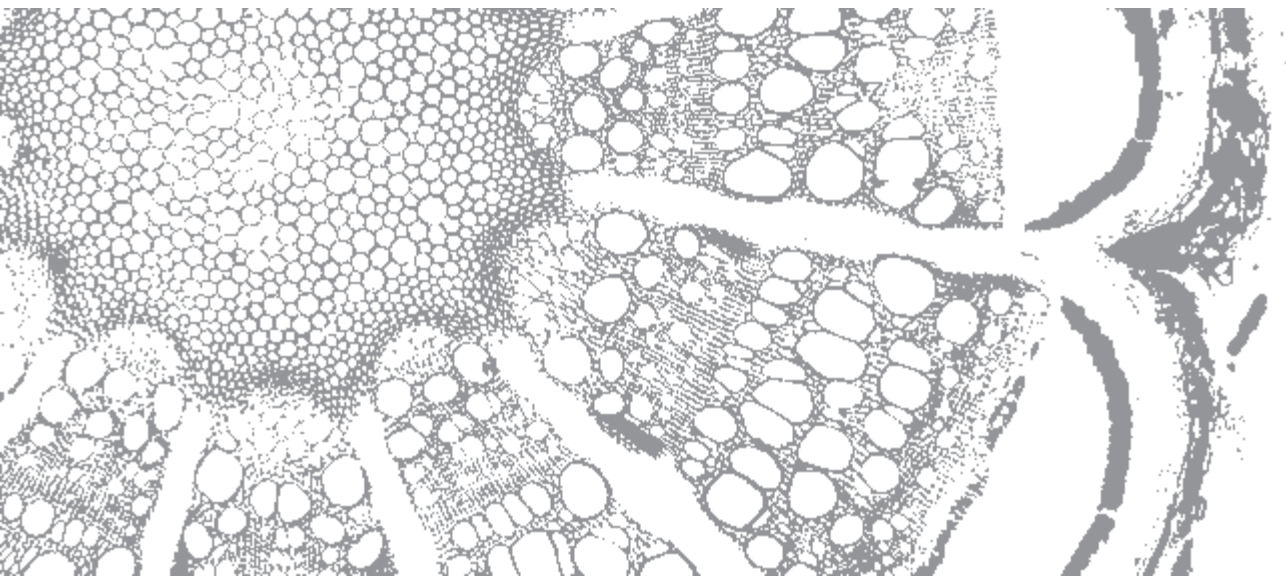


Abstract

At present there is an increasing interest for plant ingredients and their use in drugs, for teas or in food supplements. The present review describes the nature and mechanism of action of the phytochemicals presently receiving increased attention in the field of food toxicology. This relates to compounds including aristolochic acids, pyrrolizidine alkaloids, beta-carotene, coumarin, the alkenylbenzenes safrole, methyleugenol and estragole, ephedrine alkaloids and synephrine, kavalactones, anisatin, St. John's wort ingredients, cyanogenic glycosides, solanine and chaconine, thujone, and glycyrrhizinic acid. It can be concluded that several of these phytotoxins cause concern, because of their bioactivation to reactive alkylating intermediates that are able to react with cellular macromolecules causing cellular toxicity, and, upon their reaction with DNA, genotoxicity resulting in tumors. Another group of the phytotoxins presented is active without the requirement for bioactivation and, in most cases these compounds appear to act as neurotoxins interacting with one of the neurotransmitter systems. Altogether the examples presented illustrate that natural does not equal safe and that in modern society adverse health effects, upon either acute or chronic exposure to phytochemicals, can occur as a result of use of plant- or herb-based foods, teas or other extracts.

Introduction

Plants and their constituents have been used for ages as a source of bioactive ingredients for hunting, medical, warfare and assassination purposes. The Ebers papyrus (circa 1500 BC) already described a variety of poisons based on plant ingredients [1]. The ancient Greek and Roman literature gives several references to the use of poisons. Hemlock, which contains the nicotinic acid agonist alkaloids coniine and γ-conicine as its major toxic ingredients, was the official Greek state poison used for the execution of Socrates (470-399 BC) [1]. The use of plants and their ingredients for beneficial health purposes continues from these ancient times, through the Middle Ages, when botanical gardens were established and maintained as a source of medical plants, till modern time. At present there is an increasing interest for plant ingredients and their use in drugs, for teas or in food supplements. Many consumers equate "natural" with "safe" when considering plant-based food supplements or drug preparations. Unfortunately, the assumption that natural products are safe is false. Scientific literature describes a wide variety of plant-derived toxins, known as phytotoxins, which can be present in the fruit and vegetable components of our diet. These bioactive ingredients are generally regarded as safe (GRAS) at the current levels of exposure. However, in spite of a long history of safe use, botanical or herb-based food items may contain individual ingredients known to be toxic and even genotoxic and carcinogenic, and they may become of concern upon increased exposure. The present review describes the nature and mechanism of action of the phytochemicals presently receiving increased attention in the field of plant and herb-based food items. This relates to the mechanism of action and toxic effects of compounds including aristolochic acids, pyrrolizidine alkaloids, beta-carotene, coumarin, the alkenylbenzenes safrole, methyleugenol and estragole, ephedrine alkaloids and synephrine, kavalactones, anisatin, St. John's wort ingredients, cyanogenic glycosides, solanine and chaconine, thujone and glycyrrhizinic acid.

Review

In the following sections the molecular mechanisms of toxicity of a series of phytotoxins of present interest in the field of food toxicology are summarized. Emphasis is on the molecular mechanisms underlying the toxicity. Compounds discussed have been selected because of their impact in the field of food toxicology during the past decade.

Aristolochic acids

Major characteristics, occurrence and intake

Aristolociaceae have been used since ancient times in herb-based medicine. In 1991, a unique form of nephropathy was reported in Belgium. Over 100 young women suffered from kidney damage, developing in several patients into cancer of the kidneys and the urinary tract [2, 3]. This adverse effect was associated with the prolonged intake of a Chinese herb-based weight loss preparation in which *Stephania tetranda* was accidentally

replaced by *Aristolochia fanchi*, because both plants are used under the same name *'Fangji'* in Chinese folk medicine [2]. Aristolochic acids occur throughout the plants and appear in roots, stem, leaves and fruit of *Aristolochia fanchi*. Levels in the crude drug can range between 0.1% and 0.6% dry weight [4, 5]. The clinical symptoms observed were named Chinese Herb Nephropathy (CHN) [3], and when it became clear that they were caused by aristolochic acids the disease was also named Aristolochic Acid Nephropathy (AAN) [6]. The ingested dose of aristolochic acids by patients with CHN has been estimated to be in the range of a few µg/kg bw/day [7].

Mechanism of toxic action

Aristolochic acids I and II (Figure 1) are the major type of aristolochic acids and are know to be nephrotoxic, genotoxic and carcinogenic [8-13]. The first symptoms of AAN are the excretion of low molecular weight proteins in urine, indicating a toxic effect on the proximal tubules [14]. Reductive metabolic activation of aristolochic acids by cytochromes P450 (CYP) 1A1 or 1A2, and/or by other enzymes including NADPH:P450 reductase, xanthine oxidase, NAD(P)H: quinone oxidoreductase (= DT-diaphorase), and peroxidases has been reported and results in formation of a cyclic reactive nitrenium ion (Figure 2) able to form covalent DNA adducts at the exocyclic amino groups of guanine and adenosine [8, 9, 15-20]. The adduct detected with highest frequency is the adenine adduct leading to an AT to TA transversion. The human tumor suppressor gene p53 was shown to be a hot spot for this type of mutation by aristolochic acid metabolites [9]. Furthermore, in rodents the activation of the Ha-*ras* gene by a specific AT to TA transversion mutation was reported [21, 22].

Studying 39 women with CHN, Nortier et al. [23] demonstrated aristolochic acid related DNA adducts in specimens of renal tissue, and concluded that a cumulative dose of 201 g or more of a compound labeled as containing *Stephania tetrandia* but actually containing *Aristolochia fangchi* increases the risk for developing urothelial carcinomas. This was concluded based on the observation that among the 24 patients with CHN that consumed a total dose of 200 or less, 8 cases of urethelial cancer were detected, whereas among 15 patients who had ingested 201 g or more 10 cases were found, which was significantly higher (P=0.05). Since the first reports of CHN in Belgium, similar cases have been described in several other countries including Spain, Japan, France, the UK and China [9 and references therein].

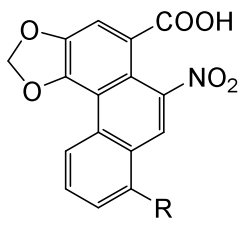


Figure 1 Structural formula of aristolochic acids [8-13]; with R=OCH_3 in aristolochic acid I (8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid, and R= H in aristolochic acid II (6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid

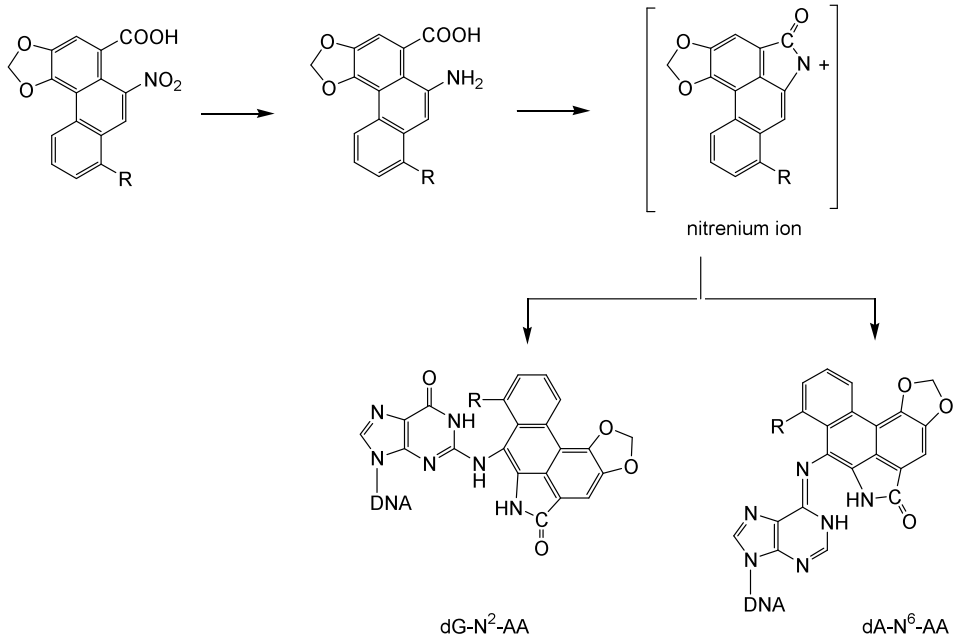


Figure 2 Metabolic activation of aristolochic acids to a cyclic reactive nitrenium ion and its subsequent covalent adduct formation with deoxyadenosine (dA-N^6-AA) and deoxyguanosine (dG-N^2-AA); reductive activation of aristolochic acids in the first step can be catalyzed by CYP1A1 or CYP1A2, and/or by other enzymes including NADPH:P450 reductase, xanthine oxidase, NAD(P)H: quinone oxidoredutase (= DT-diaphorase), and peroxidases [8, 9, 15-20]

Polymorphisms of influence

To date, only a few percent of the patients treated with the slimming regimen are reported to have suffered from nephropathy [20]. A possible explanation for this observation may be differences in the enzymes involved in bioactivation/ detoxification of the aristolochic acids. Genetic polymorphisms in the enzymes catalyzing reductive activation, including CYP1A1 or CYP1A2, but also NADPH:P450 reductase, xanthine oxidase, NAD(P)H:quinone oxidoreductase (= DT-diaphorase), and peroxidases may be of influence. Thus, lifestyle factors like smoking, which induces CYP1A, and genetic polymorphisms for CYP1A2 and for NAD(P)H:quinone oxidoreductase leading to poor metabolizer phenotypes, may influence the risk posed by aristolochic acid consumption.

Concluding remarks

Following the reports of *Aristolochia*-related nephrotoxicity many countries have taken regulatory actions to protect the public by taking Aristolochia species from the supply chain. The European Agency for the Evaluation of Medicinal products even suggested to consider the prohibition of species at risk of being confused with *Aristolochia* species, unless appropriate quality control procedures are in place [7]. Such species include *Akebia quinata, Akebia trifoliata, Clematis armandii, Clematis montana, Cocculus orbiculatus, Cocculus*

laurifolius, Cocculus trilobus and Stephania tetrandra [7]. It can also be concluded that further identification of the enzymes principally involved in the bioactivation of aristolochic acids, and the screening of CHN patients for genetic polymorphisms in the major enzymes involved, seem important future steps to allow elucidation of possible relationships between genotypes and CHN, and to define the groups within the human population at increased risk.

Pyrrolizidine alkaloids (PAs)

Major characteristics, occurrence and intake

Plants known to contain pyrrolizidine alkaloids (PAs) are widely used for medicinal purposes as home remedies all over the world, and some are even used as food. Human poisoning and even deaths from PAs have been reported in several countries including South Africa, Jamaica, Ecuador, Hong Kong, India, the former central Asian republics of the USSR, the UK and the US [24, 25]. PAs are present in plants of the families *Boraginaceae* (all genera), *Asteraceae* (*Senecioneae* and *Eupatorieae*), and *Fabaceae* (genus *Crotalaria*) [24]. PAs were known to be a hazard towards livestock for many decades. Human consumption of PAs occurs, for example, from consumption of *Symphytum* and *Senecio* species present in herbal preparations, such as "comfrey tea" or "groundsel tea". Over 200 PAs have been identified, about half of them estimated to be toxic.

Figure 3 presents the structures of some important pyrrolizidine alkaloids including echimidine, the most toxic PA in *Symphytum officinale*; jacobine, the major toxic alkaloid in *Senecio jacobaea*, and retrorsine, the major toxic alkaloid in *Senecio vulgaris*. *Symphytum officinale*, commonly called comfrey, contains in addition to echimidine also other alkaloids, including intermedine, lycopsamine, symphytine, and symglandine. The total content of PAs is nearly 0.5% in *Symphytum caucasicum*, but lower in *S. officinale* (leaves: 0.02-0.18%; roots: 0.25-0.29%) and *S. peregrinum* was found to contain about 0.2% alkaloids in the tops [26]. Aside from ingesting the plants directly, PAs can be consumed by eating honey collected by bees that visit PA-containing plants (mainly species of *Senecio*) and by drinking milk or eating eggs produced by animals that have consumed PA-containing plants [26-29]. In honey originating from species of *Senecio*, the total concentration of PAs was 0.3-3.2 µg/kg. PAs could be detected in the concentration range of 30-70 µg/kg in honey from the Alpine foothills of Switzerland [26, 27]. The range of toxic doses in humans is about 0.1-10 mg/kg bw/day [26, 29]. However, the World Health Organization (WHO) suggested that the lowest intake of PAs that caused adverse effects in a human was just 0.015 mg/kg bw/day, corresponding to 1 mg/day for a 70 kg adult, based on the use of comfrey [24, 26]. Exposure to PAs can vary since PA content of comfrey roots and leaves have been reported to vary between 450 – 8300 mg/kg for roots and between 15-55 mg/kg for leaves [26, 30].

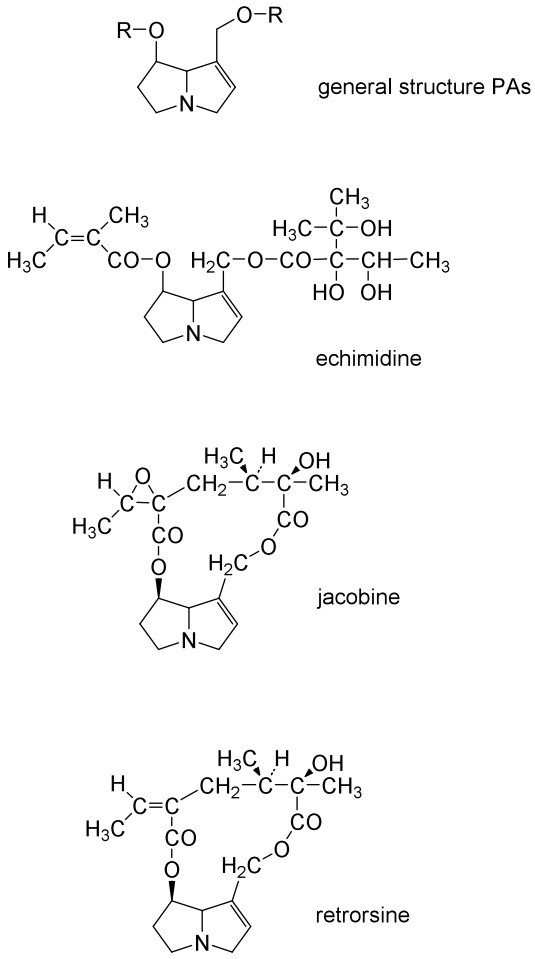


Figure 3 Schematic presentation of the general structure of pyrrolizidine alkaloids (PAs), and structural formula of some important PAs including echimidine, the most toxic PA in *Symphytum*, retrorsine, the major toxic alkaloid in *Senecio vulgaris* and jacobine, the major toxic alkaloid in *Senecio jacobaea*; the hepatotoxic alkaloids have a 1,2-double bond in the pyrrolizidine ring and branched side chains, in which the 9-hydroxyl and preferably also the 7-hydroxyl substituent are esterified [24]

Mechanism of toxic action

The toxic effects of PAs in humans are principally on the liver. Here they can produce veno-occlusive disease (VOD), the major lesion being the occlusion of the central and sublobular hepatic veins [24]. The PAs are also known to cause liver damage in experimental and farm animals [24]. Furthermore, PAs are known to be mutagenic, carcinogenic and teratogenic [24]. The mechanism of toxic action has been related to the formation of pyrrole-type metabolites [24, 31, 32]. These pyrrolizidine pyrroles are pyrrolic dehydro-alkaloids, formed by dehydrogenation of the pyrrolizidine alkaloids by hepatic monooxygenases, especially CYPs (Figure 4) [24, 32]. The formation of the pyrrolic metabolites results from an initial hydroxylation of the unsaturated pyrrolizidine ring

adjacent to the nitrogen atom, leading to an unstable intermediate that decomposes to give the pyrrolic product [33]. The pyrrolic dehydro-alkaloid metabolites are reactive alkylating agents able to react with cellular nucleophiles (Figure 4), thereby causing DNA alkylation, DNA crosslinking and liver cell necrosis [24 and references therein]. The carcinogenic activity of PAs appears to parallel their mutagenic behavior but not their hepatotoxicity [24]. In addition, upon release from the liver the pyrroles may also affect the endothelium of blood vessels in the liver or lungs. Hydrolysis of the ester groups of PAs by esterases and formation and excretion of water soluble N-oxides are detoxification mechanisms, although for some PAs N-oxidation may represent a bioactivation pathway [32-34]. Conjugation by cellular nucleophiles including especially glutathione is an important detoxification pathway for the pyrrolic dehydro-alkaloid metabolites [32, 33 and references therein].

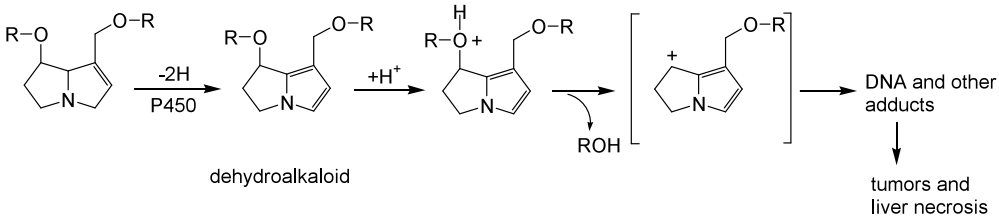


Figure 4 Metabolic activation of pyrrolizidine alkaloids to pyrrolic dehydro-alkaloids, proceeding by an initial hydroxylation of the unsaturated pyrrolizidine ring adjacent to the nitrogen atom by cytochromes P450, leading to an unstable intermediate that decomposes to give the pyrrolic dehydro-alkaloid product that reacts further to an alkylating intermediate [24]

Polymorphisms of influence

Sheep, guinea pigs, gerbils, rabbits, hamsters and Japanese quail are highly resistant to pyrrolizidine alkaloid toxicity, whereas rats, cattle, horses and chickens are highly susceptible [32]. These species dependent differences in sensitivity towards pyrrolizidine alkaloids have been related to species dependent differences in the conversion of the PAs to their pyrrole metabolites, although species dependent differences in detoxification mechanisms may also play a role [32, 34]. Metabolism of PAs to dehydropyrrolizidines is catalyzed by especially CYP3A and CYP2B6 isoenzymes [34]. Life style factors and genetic polymorphisms known to occur in the CYP3A family may play a role in interindividual differences in sensitivity towards PAs. This includes for example induction of CYP3A4, the most important drug-metabolizing CYP in human liver, by drugs like rifampicin, dexamethasone and phenobarbital.

Detoxification of the PAs to the corresponding N-oxides is catalyzed by CYPs, including CYP3A, and flavin containing monooxygenase [34], and detoxification of the pyrrolic metabolites by glutathione conjugation is catalyzed by glutathione S-transferases (GSTs). The human CYP and GST isoenzymes involved in these detoxification pathways largely remain to be elucidated. Elucidation of the CYPs and of the GST isoenzymes involved in the detoxification pathways of PAs is required to conclude what other life style factors and genetic polymorphisms are likely to influence the interindividual differences in sensitivity.

Concluding remarks

An intake of 1 mg PAs /day, the lowest dose reported to cause veno-occlusive disease in a human, would be reached upon intake of 0.7 grams of a herb-based preparation that contains 1520 mg/kg. This is an amount that may be found in capsules on the market, indicating that such a product would be too toxic to consume on a regular basis. On the other hand products with no detectable PAs are also encountered. Together this indicates that regulatory actions are required until appropriate quality control procedures are in place.

Beta-carotene

Major characteristics, occurrence and intake

In industrialized countries, fruits and vegetables provide an estimated 1.7-3 mg/day of pro-vitamin A carotenoids, of which beta-carotene is the principal component [35]. Other sources of beta-carotene include food additives (1-2 mg/person/day) and supplements [35]. Carotenoids, including beta-carotene and others, possess antioxidant and radical scavenging ability [36-39]. However, experimental studies with beta-carotene, at present provide perhaps the best example of unexpected health risks related to increased intake levels of a bioactive plant ingredient. Observational epidemiologic studies indicate that diets high in carotenoid-rich fruits and vegetables as well as increased serum levels of beta-carotene are associated with a decreased risk of lung cancer [40-42]. Based on these observations large human intervention trials with heavy smokers receiving beta-carotene supplements were undertaken [43, 44]. The studies reported increased, instead of decreased levels of lung cancer incidence in the population of heavy smokers receiving beta-carotene supplements for several years. Similar to the effects of beta-carotene on lung cancer risk in heavy smokers, an increased lung cancer risk due to beta-carotene supplementation in asbestos-exposed workers was also reported [43]. More recently, Baron et al. [45] reported increased risk on colon cancer in cigarette smokers with a high intake of beta-carotene.

Mechanism of toxic action

The mechanism by which beta-carotene increases lung cancer risk in both heavy smokers and asbestos workers is at present unclear, although some hypotheses and initial results have been reported. One possible mechanism is a co-carcinogenic effect of beta-carotene mediated through a stimulating effect of beta-carotene on phase I bioactivating enzymes. Induction of CYP activity by beta-carotene, in particular of CYP1A1 and CYP1A2 (activating aromatic amines, polychlorinated biphenyls, dioxins and PAHs), CYP2A (activating butadiene, hexamethyl phosphoramide and nitrosamines), CYP2B1 (activating olefins and halogenated hydrocarbons) and CYP3A (activating aflatoxins, 1-nitropyrene and PAHs), may result in increased formation of genotoxic metabolites of, amongst others, cigarette smoke constituents [35]. Another possible explanation suggests that high dose beta-carotene supplementation may enhance lung tumorigenesis in smokers by altering retinoid signaling. This may proceed through the formation of reactive oxidative cleavage products of beta-carotene that are able to interfere with normal retinoid signaling. The high oxygen pressure in the lungs may favor this oxidative

degradation of beta-carotene [46]. In addition, the interaction between reactive oxygen species, derived from tobacco smoke or induced in the lung upon asbestos exposure, may result in beta-carotene oxidation, which could lead to these toxic beta-carotene metabolites [47-50]. Reduction of retinoid signaling could also occur after induction of CYPs, CYP1A1 or CYP1A2 in particular, by cigarette smoke and high doses of beta-carotene, resulting in enhanced retinoic acid catabolism in the lung [51]. A hypothesis explaining how disturbed retinoid signaling may result in the increased lung tumor risk is presented in Figure 5. Alterations in retinoid signaling could result in reduced retinoid levels and suppression of RARß gene expression, the latter representing a tumor suppressor gene [35, 48-51]. Furthermore the whole process may induce increased expression of c-jun and c-fos genes resulting in higher levels of activator protein-1 (AP-1). Increased expression of c-Jun and c-Fos proteins has been reported for several mitogenic stimuli and tumor-promoting agents, and has been observed in tobacco-smoke exposed ferrets supplemented with high-dose beta-carotene [50, 51].

Of importance to note is that these tumor-promoting effects of beta-carotene are especially observed upon high dose supplementation in heavy smokers. Beta-carotene does not exert this tumor risk enhancing effect in former smokers [43]. In asbestos workers beta-carotene oxidation may be stimulated by the inflammatory process known to be induced in asbestos-exposed lungs [47]. Inflammatory cells isolated from nonsmokers with asbestosis are known to release significantly increased amounts of reactive oxygen species compared to cells recovered from control individuals [52].

Polymorphisms of influence

Depending on the actual mechanism underlying the adverse effect of beta-carotene in heavy smokers, different polymorphisms may be of influence. When the induction of CYPs involved in bioactivation of pro-carcinogens proves to be an important mechanism underlying the adverse effect in smokers, genetic polymorphisms in CYP1A1, CYP1A2, CYP2A, CYP2B1 and CYP3A may be of relevance. A role for increased retinoid acid catabolism has been related to the activity of especially CYP1A1 or CYP1A2. This implies that genetic polymorphisms modifying the activity of these two CYPs may influence the sensitivity of heavy smokers to the adverse effects of beta-carotene.

Concluding remarks

Recently the SCF concluded that there might be a very small difference between the levels of beta-carotene that may confer health benefits (up to 10 mg/day from especially natural sources), and those that may produce adverse effects in smokers (20 mg/day) [35]. Therefore, beta-carotene suppletion should be regarded with caution. Furthermore, the role of other carotenoids in the reported association between reduced incidence of lung cancer and increased intake of vegetables and fruits rich in carotenoids remains to be elucidated [35]. The SCF also concluded that the possibility that some of the oxidative cleavage products of beta-carotene could interfere with retinoic acid homeostasis requires further investigation [35].

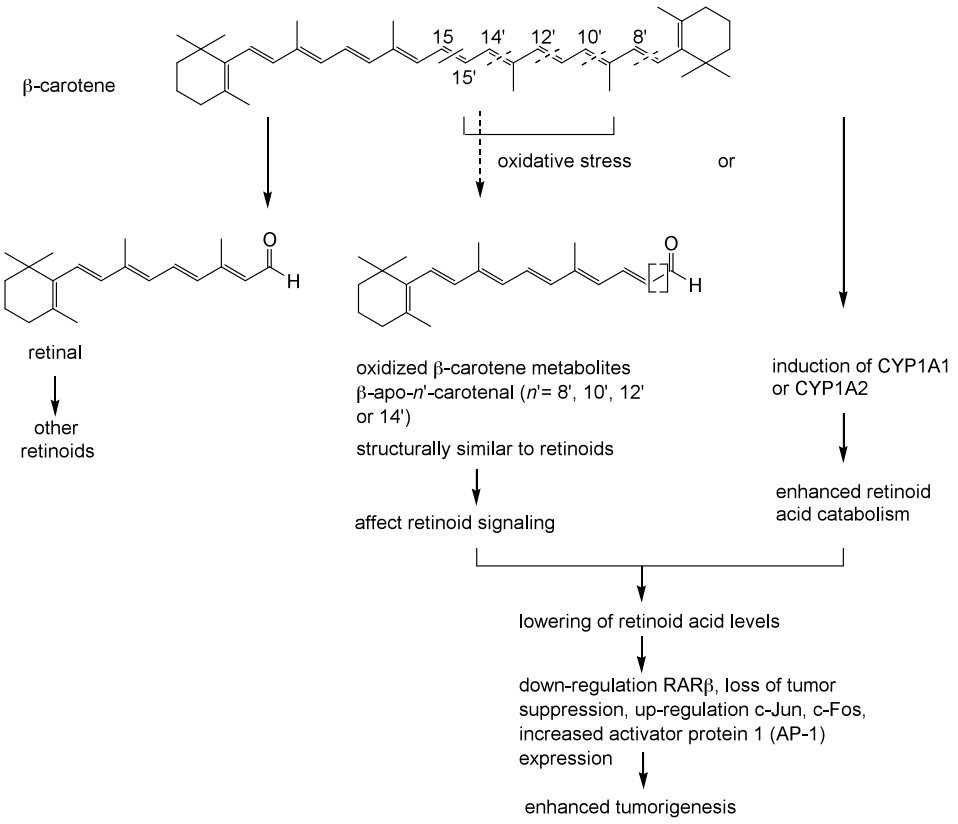


Figure 5 Hypotheses for the oxidative mechanism for tumor enhancement by beta-carotene [46-51]

The possible role of reduced retinoid acid levels in the mechanism of beta-carotene-mediated enhancement of cigarette smoke-induced lung cancer, has led some investigators to the conclusion that perhaps restoration of lung retinoic acid homeostasis by retinoic acid supplementation or by inhibition of CYP-enhanced retinoid acid catabolism can have chemopreventive effects against lung carcinogenesis [51]. This is an interesting hypothesis that needs further study.

Coumarin

Major characteristics, occurrence and intake

Coumarin is a constituent of cinnamon, an important food flavor. Coumarin is naturally found at high levels in some essential oils, such as cinnamon bark oil (7000 mg/kg), cinnamon leaf oil (40,600 mg/kg), cassia leaf oil (up to 83,300 mg/kg), and lavender and peppermint oil (20 mg/kg) [53]. It is also found in some fruits (bilberry; 0.0005 mg/kg), green tea (1.2-1.7 mg/kg) [53] and other foods, such as chicory, honey cinnamon cake and in 'speculaas', a Dutch sweet spicy biscuit (35 mg/kg) [54]. The average human exposure to coumarin from

the diet and from fragrance use in cosmetic products is about 0.06 (i.e. 0.02 + 0.04) mg/kg bw/day [54, 55]. The use of coumarin as a food flavor was discontinued as a result of the finding of hepatotoxic effects in rats and dogs fed with coumarin in the diet [54-56]. Coumarin was banned in the USA in 1954 based on reports of hepatotoxicity in rats, prior to the existence of any carcinogenicity and mutagenicity data, and was recommended for withdrawal from use in the UK in 1965. In 1999, the EU Scientific Committee on Food (EU-SCF) listed coumarin as an 'active principle' and set the maximum permitted concentration for coumarin in food and alcoholic beverages at 2 mg/kg [54]. Enforcement of this concentration means the withdrawal of many products from the market.

Mechanism of toxic action

Pharmacokinetic studies in humans have demonstrated that coumarin is completely absorbed from the gastrointestinal tract after oral administration and extensively converted by first pass metabolism in the liver, with only between 2 and 6% reaching the systemic circulation intact [55]. In the rat, a relatively large amount is excreted via the bile. Thus, an appreciable proportion of the dose is excreted in the feces. The urine appears to be the major route of excretion in humans. Based on animal data the EU-SCF [54] concluded that coumarin is a carcinogen via the oral route, resulting in adenomas and carcinomas of the liver and bile ducts and adenomas of the kidney in rats, and in adenomas and carcinomas of the lung and adenomas of the liver in mice.

The major characteristics of coumarin metabolism are presented in Figure 6. The pathway leading to 7-hydroxylation is considered a detoxification pathway and the pathway leading to formation of a coumarin 3,4-epoxide intermediate is the toxic bioactivating route. A number of studies have demonstrated that both acute and chronic coumarin-induced liver injury in the rat appears to be due to the presence of the 3,4-double bond and that the first step in coumarin bioactivation involves the CYP-dependent formation of a 3,4-epoxide intermediate [54-56]. A key issue in the recent risk assessment of coumarin has been the question of whether or not coumarin is genotoxic. Recently, the European Food Safety Authority (EFSA) reevaluated coumarin and concluded that data on the absence of DNA adduct formation in kidney and liver of coumarin-exposed rats indicate that coumarin does not bind covalently to DNA *in vivo*. These results suggest that coumarin induces tumors via a mechanism of action that is preceded by toxicity in the target organ and that this will be reflected in a dose-response curve with a threshold reflecting a no adverse effect level [53]. This conclusion was corroborated by the facts that coumarin does not induce unscheduled DNA synthesis in male SD rat hepatocytes [57] and that coumarin was negative in an *in vivo* micronucleus assay in mice [58]. Based on this threshold type dose-response curve for tumor formation, EFSA suggested a TDI (Tolerable Daily Intake) for coumarin of 0-0.1 mg/kg bw/day [53]. The mechanism of this coumarin toxicity remains to be established, but metabolites down the 3,4-epoxidation pathway could play a role [53-56, 59]. Coumarin 3,4-epoxide can either rearrange spontaneously to *o*-hydroxyphenylacetaldehyde (*o*-HPA) or conjugate with glutathione, the latter either chemically or catalyzed by GST-alpha or GST-mu, but not GST-pi enzymes (Figure 6) [59].

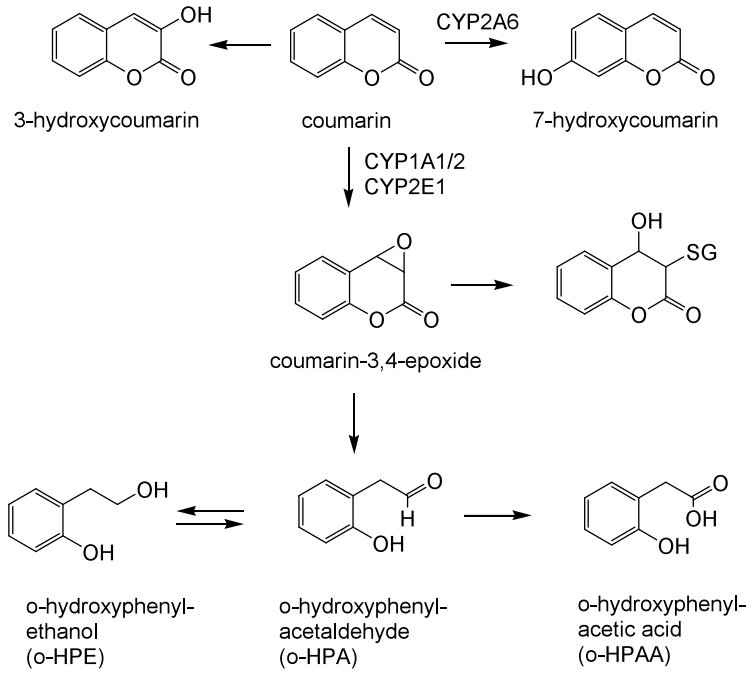


Figure 6 Biotransformation reactions of coumarin leading to detoxification (7-hydroxylation and 3-hydroxylation) or metabolic activation via the coumarin 3,4-epoxide pathway [55]; formation of coumarin 3,4-epoxide is catalyzed by especially CYP2E1, and, to a lower extent, by CYP1A1 and CYP1A2 [62]; 7-Hydroxylation is catalyzed by CYP2A6 [55]; coumarin 3,4-epoxide can either rearrange spontaneously to *o*-hydroxyphenylacetaldehyde (*o*-HPA) or conjugate with glutathione, the latter either chemically or catalyzed by GST-alpha or GST-mu, but not GST-pi enzymes [57]; *o*-HPA is hepatotoxic [60] and is further converted by oxidation to *o*-hydroxyphenylacetic acid (*o*-HPAA) or by reduction to *o*- hydroxyphenylethanol (*o*-HPE)

o-HPA is hepatotoxic [60] and is further converted by oxidation to *o*-hydroxyphenylacetic acid (*o*-HPAA) or by reduction to *o*-hydroxyphenylethanol (*o*-HPE) (Figure 6) [55, 59]. Oxidation to *o*-HPAA is considered a detoxification step coupled to urinary excretion, whereas reduction to *o*-HPE is followed by oxidation back to *o*-HPA thereby contributing to slower hepatic clearance of the hepatotoxic *o*-HPA [59]. Detoxification of *o*-HPA to *o*-HPAA may even be the major determinant of species differences in coumarin-induced hepatotoxicity [59].

Polymorphisms of influence

The extent of coumarin 7-hydroxylation appears to be species rather than dose dependent [55]. The major pathway of coumarin metabolism in the rat is 3,4-epoxidation, 7-hydroxylation being a minor route. The 3,4-epoxidation is also the major route of coumarin metabolism in the mouse, although major sex and strain differences exist. It was demonstrated that coumarin was more toxic for C3H/HeJ than for DBA/2J mice [61]. This might be explained by the fact that DBA/2J strain mice have higher hepatic coumarin 7-hydroxylation than C3H/HeJ strain mice.

Unlike the rat and the mouse, where the 3,4-epoxidation pathway predominates, the major pathway of coumarin metabolism in humans is 7-hydroxylation. This might explain why there is little evidence of coumarin-induced toxicity in humans given therapeutic doses of coumarin that are up to 1900 times higher than those obtained from dietary sources and from fragrances used in cosmetic products. In the majority of human subjects studied, coumarin is extensively metabolized in the liver to 7-hydroxycoumarin by CYP2A6, although humans can also metabolize coumarin by 3,4-epoxidation [54-56, 62]. Studies using rat and human recombinant CYP enzymes have pointed at the formation of coumarin 3,4-epoxide by especially CYP2E1, and, to a lower extent, by CYP1A1 and CYP1A2 in both species [62].

There appears to be a marked interindividual variation in coumarin metabolism to 7-hydroxycoumarin in humans due to a genetic polymorphism that exists in human CYP2A6 [55, 63, 64]. The role of CYP2A6 polymorphism in human risk profiles for coumarin remains to be elucidated. At present it is still unknown which pathway takes over the metabolism of coumarin in individuals with the CYP2A6 deficiency. It cannot be ruled out that the pathway taking over is 3,4-epoxidation, also because CYPs other than CYP2A6, such as CYP2A13, have been reported to be able to catalyze both coumarin 7-hydroxylation but also the formation of metabolites representative for the 3,4-epoxide route, both to a comparable extent [65].

Recently, the CYP2A6 genotype and development of hepatotoxicity in patients who were dosed with 90 mg coumarin/day have been evaluated. From 231 patients 16 appeared to be defective for the CYP2A6 genotype, being heterozygous for the CYP2A6*2 allele that leads to an inactive protein. Of the nine patients showing evidence of hepatotoxicity only one had the variant allele, eight being wild-type homozygotes [66]. This result indicates that a single copy of a variant CYP2A6 allele does not confer susceptibility to liver dysfunction in patients treated with coumarin [66]. Since the conversion of *o*-HPA to *o*-HPAA is catalyzed by aldehyde dehydrogenase, polymorphisms known to occur in this enzyme could contribute to interindividual differences in sensitivity toward coumarin induced toxicity, although this remains to be demonstrated.

Concluding remarks

Recently, the European Food Safety Authority (EFSA) reevaluated coumarin and concluded that coumarin induces tumors via a mechanism of action that is preceded by toxicity in the target organ [53]. Based on this, EFSA suggested a TDI (Tolerable Daily Intake) for coumarin of 0.1 mg/kg bw/day [53]. The estimated theoretical maximum daily intake of coumarin via food is 4.085 mg/day (0.07 mg/kg bw/day) or lower (1.3-1.5 mg/day which equals 0.02 mg/kg bw/day), given a more realistic intake scenario [53, 55]. These intake scenarios are below the TDI, suggesting that withdrawal of products from the market would no longer be an issue.

Alkenylbezenes: safrole, methyleugenol and estragole

The group of alkenylbenzenes includes compounds like safrole, methyleugenol and estragole (Figure 7), which are important constituents of herbs like nutmeg, cinnamon, anise star, tarragon, sweet basil, sweet fennel and anise vert. The EU-SCF has launched scientific evaluations on these three alkenylbenzenes [67-69]. The

EU-SCF concluded that safrole, methyleugenol and estragole are genotoxic and carcinogenic, and indicated restrictions in use. Recently, however, an industrial expert panel from the Flavor and Extract Manufacturers Association (FEMA) published that exposure to methyleugenol and estragole, resulting from spice consumption, does not pose a significant cancer risk for humans [70]. The mechanistic argument underlying this conclusion relates to insight in the toxicokinetics and the mechanism of genotoxicity of the alkenylbenzenes.

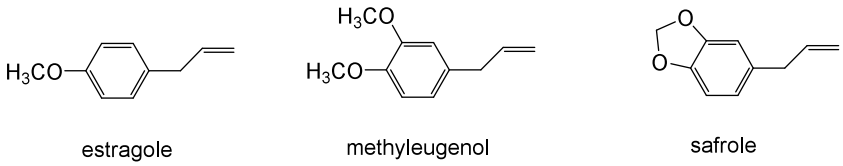


Figure 7 Structure of the alkenylbenzenes safrole, methyleugenol and estragole

Alkenylbezenes: estragole

Major characteristics, occurrence and intake

Estragole occurs naturally in a variety of foods including tarragon (60-70% of essential oil), sweet basil (20-43% of essential oil), sweet fennel (5-20% of essential oil), anis vert (1% of essential oil), and anis star (5-6% of essential oil [69]. There are several food categories to which estragole could be added. For alcoholic beverages, canned fish, and fats and oils, estragol levels may amount to 100 mg/kg (approximately 4% of the market share), 50 mg/kg (approximately 30% of the market share) and 250 mg/kg (approximately 1% of the market share), respectively [69]. Based on these assumptions, the average daily intake from food was estimated to amount to 4.3 mg/person/day and the 97th percentile to 8.7 mg/person/day [69].

Mechanism of toxic action

Figure 8 presents an overview of the relevant metabolic pathways of estragole, which is also representative for the metabolic profiles of methyleugenol and safrole. The CYP-derived metabolite 1'-hydroxyestragole is the putative proximate carcinogen of estragole. It has been found in the urine of men dosed with 1 µg estragole/kg bw [71]. The ultimate electrophilic and carcinogenic metabolite of estragole is formed as a result of sulfotransferases converting 1'-hydroxyestragole to 1'-sulfooxyestragole [72]. Another major metabolic pathway of estragole in rats, mice and humans includes O-demethylation. At higher doses the proportion of O-demethylation falls and the pathway leading to formation of 1'-hydroxyestragole increases (from 1.3-5.4% of the dose in the range of 0.05-50 mg/kg bw to 11.4-13.7% in the dose range of 500-1000 mg/kg bw for rats and mice) [73, 74]. Thus, it appears that the 1'-hydroxylation pathway is more prominent at higher levels of exposure.

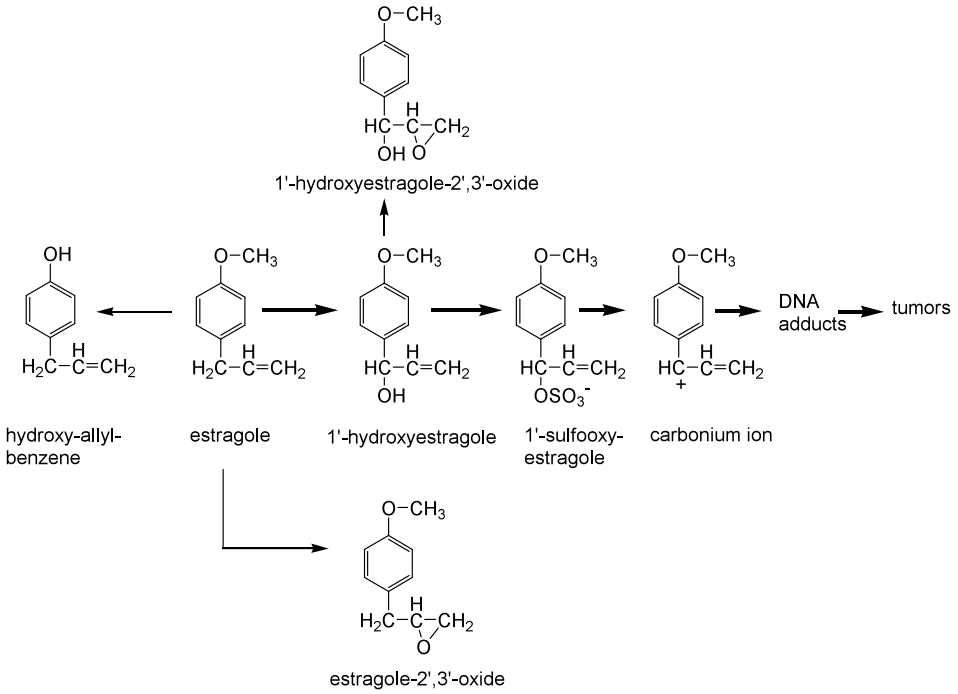


Figure 8 Metabolic pathways for bioactivation and detoxification of estragole (also relevant for methyleugenol and safrole) [67-69]

The FEMA USA Expert Panel even concluded that at low dose (100 µg/person which amounts to 1.5 µg/kg bw), human production of the 1'-hydroxy metabolite is expected to be very low given that urinary excretion of the 1'-hydroxy metabolite is below 0.5% of the dose administered. This relative decrease in conversion to the proximate carcinogenic metabolite at lower doses, was an important argument for the FEMA USA Expert Panel to conclude that exposure to methyleugenol and estragole resulting from spice consumption does not pose a significant cancer risk for humans [70].

In addition to formation of 1'-hydroxyestragole, formation of estragole-2',3'-oxide and 1'-hydroxyestragole-2',3'-oxide (Figure 8) provide possible additional bioactivation pathways of estragole [75]. The estragole metabolites estragole-2',3'-oxide and 1'-hydroxyestragole-2',3'-oxide have been shown to be hepatocarcinogenic [72, 76] and to produce DNA adducts *in vitro* [77-79]. However, adducts of these 2',3'-oxides were not among the major adducts found in mouse liver following *in vivo* administration of estragole [79]. Therefore it is concluded that they do not contribute significantly to the genotoxicity of estragole. The apparent absence of a role for estragole epoxidation in the genotoxicity of estragole has been ascribed to very rapid and efficient detoxification of the 2',3'-oxides in the cell by a combination of epoxide hydrolases and glutathione S-transferases [75].

Polymorphisms of influence

Human CYPs involved in the bioactivation of estragole to 1'-hydroxyestragole have not been identified. They may be in line with CYPs found to be involved in bioactivation of the related alkenylbenzenes methyleugenol and safrole, discussed below. Glucuronidation of 1'-hydroxyestragole catalyzed by uridine diphosphate glucuronosyltransferase (UGT) isoenzymes is a detoxification pathway and was recently shown to be catalyzed by human UGT2B7, UGT1A9 and UGT2B15 [80]. The UGT2B7 polymorphisms, leading to slow glucuronidators, may potentially lead to differences in toxicity of estragole. Life style factors, like concomitant chronic intake of therapeutic drugs and dietary components which increase the levels of UGT2B7 expression or which are substrates for UGT2B1 or UGT1A9, might also modify the relative risk [80]. Human CYPs of relevance for the detoxification pathways, and the type of sulfotransferases involved in the bioactivation to the ultimate electrophilic and carcinogenic metabolite 1'-sulfooxyestragole remain to be elucidated.

Concluding remarks

The EU-SCF concluded that estragole is genotoxic and carcinogenic, and indicated restrictions in use [74]. This will result in restrictions in use for the compound itself, but not for estragole containing herb extracts. Improved risk extrapolation from high dose animal experiments to low dose carcinogenic risks in man, taking into account the toxicokinetics of the CYPs responsible for the different biotransformation pathways, seems to be a prerequisite for future improvement of risk estimates for this alkenylbenzene.

Alkenylbezenes: methyleugenol

Major characteristics, occurrence and intake

Methyleugenol is a natural constituent of a number of plants including nutmeg, pimento, lemongrass, tarragon, basil, star anise and fennel [68]. The compound is also used as a flavoring agent in jellies, baked goods, non-alcoholic beverages, chewing gums, relish and ice cream, and as a fragrance in several cosmetic products [68]. Intake estimates may vary widely because of lack of data about the concentration of the chemical in foodstuffs [68]. Intake estimates reported by the EU-SCF amount to an average intake for consumers of 13 mg/person/day and a 97th percentile of 36 mg/person/day [68].

Mechanism of toxic action

The metabolism and metabolic activation of methyleugenol proceeds similar to that depicted in Figure 8 for estragole. Methyleugenol and its proximate carcinogenic metabolite 1'-hydroxymethyleugenol induce liver tumors in mice and rats [76, 81]. In addition, especially at higher doses, neuroendocrine tumors of the glandular stomach, as well as renal tube hyperplasia and adenomas were observed. In vitro, methyleugenol as well as its metabolites 1'-hydroxymethyleugenol and methyleugenol-2',3'-oxide induce unscheduled DNA synthesis (UDS) in cultured rat hepatocytes [82]. Howes et al. [83] reported an excellent correlation between UDS induction in rat

hepatocytes and results from rodent carcinogenicity studies for methyleugenol and also for related alkenylbenzenes like estragole and safrole.

In addition, methyleugenol has been shown to form adducts with DNA and protein in human fibroblasts V79 cells transfected with human genes expressing sulfotransferases and in the mouse liver in vivo [84-86]. The adduct formation with methyleugenol (72.7 pmol/mg DNA) was higher than that induced by estragole (30.0 pmol/mg DNA) or safrole (14.7 pmol/mg DNA)[86]. This order is in line with the relative differences in dose regimens required to induce tumors in animal studies, known to decrease in the order safrole > estragole > methyleugenol [67-69]. These observations, together with the fact that estimated daily intakes of methyleugenol (13 mg/person/day) [68] are higher than those estimated for estragole (4.3 mg/person/day) [69] and safrole (0.3 mg/person/day) [67], seem to put the priority for risk management on methyleugenol.

Polymorphisms of influence

Recent studies revealed human CYP1A2 and CYP2C9 to be important isoenzymes in the conversion of methyleugenol to 1'-hydroxymethyleugenol, with CYP2D6 and CYP2C19 perhaps also involved in methyleugenol 1′-hydroxylation [87]. The interindividual differences found in fifteen human liver microsomes were larger (5-fold difference) than the interspecies and sex differences found in the incubations with microsomes prepared from pooled livers of male and female rats, mice, and humans (2-fold difference) [87]. Therefore, interindividual differences in methyleugenol 1′-hydroxylation seem to be at least as important as interspecies differences between rodents and humans. In particular people that smoke (induction of CYP1A2), use barbiturates (induction of 2C9), or have polymorphisms especially in the CYP2D6 gene leading to ultra rapid metabolizer phenotypes might have a higher methyleugenol 1′-hydroxylation rate. These groups of people might be at higher risk of the adverse effects of exposure to methyleugenol. Polymorphisms in CYP2C9 and CYP2D6 leading to poor metabolizer phenotypes may reduce the relative risk.

Concluding remarks.

As for estragole, methyleugenol may be converted to a variety of metabolites with different toxicological impact. Identification of the biotransformation enzymes involved in the various bioactivation and detoxification pathways and the implementation of their toxicokinetics into risk assessment models seems to be required for improved methods for extrapolation from high dose animal experiments to low dose carcinogenic risks in man.

Alkenylbezenes: safrole

Major characteristics, occurrence and intake

Similar to estragole and methyleugenol, safrole is a natural constituent of a number of spices such as nutmeg, mace, cinnamon, anise, black pepper and sweet basil. The most important dietary sources are nutmeg, mace and their essential oils. Safrole is also present in cola drinks. Safrole was the first of the class of alkenylbenzenes shown to have carcinogenic properties [88]. An exposure assessment was made based on a

selection of 28 food categories assuming a concentration of 0.5 mg safrole/kg for food in general, a concentration of 2 mg/kg for food containing cinnamon and of 5 mg/kg for food containing nutmeg. For beverages, canned fish and chewing gum the following concentrations were specified: beverages 5 mg/kg (4% of the market share), canned fish 20 mg/kg (30% of the market share), and chewing gum 10 mg/kg (2% of the market share). Using these assumptions the estimated average intake for consumers was calculated to amount to 0.3 mg/person/day and the 97th percentile to 0.5 mg/person/day [67].

Mechanism of toxic action

Chronic administration of safrole in the diet at 0.5-1.0% for a year or more caused liver tumors in adult mice and rats [72, 76, 89, 90]. The carcinogenicity of safrole metabolites, namely 1'-hydroxysafrole, safrole-2',3'-oxide and 1'-hydroxysafrole-2',3'-oxide was also clearly demonstrated [72, 76]. The main metabolic pathways are allylic hydroxylation to 1'-hydroxysafrole and oxidation and O-dealkylation, the latter leading to 4-allylcatechol that is easily oxidized to 4-allyl-o-quinone (Figure 9), and epoxidation of the allylic side chain or the aromatic ring. 1'-Hydroxysafrole and 4-allylcatechol represent the main metabolites. 1'-Hydroxysafrole can be conjugated by sulfotransferases giving rise to a conjugate that can easily split producing the ultimate carcinogenic electrophilic carbonium ion (Figure 8). In addition, oxidation of 4-allylcatechol to 4-allyl-o-quinone may also generate an electrophilic toxic metabolite [91](Figure 9). Safrole-DNA adducts were identified in livers of mice given cola beverages instead of drinking water [92]. Inhibition of both DNA adduct formation and carcinogenicity of 1'-hydroxysafrole was shown in the liver of mice deficient in synthesis of PAPS the cofactor required for sulfotransferase reactions, or mice treated with pentachlorophenol, a strong inhibitor of sulfotransferases [93].

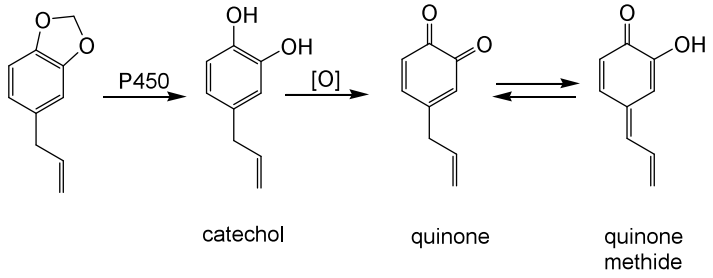


Figure 9 Alternative bioactivation pathway for safrol leading to formation of catechol and quinone-type metabolites [91]

Polymorphisms of influence

The CYP enzymes involved in the bioactivation of safrole to its proximate carcinogen 1′-hydroxysafrole in man were identified to be CYP2C9, CYP2A6, CYP2D6 and CYP2E1 [94, 95]. Data from Gentest microsomes in which the activities towards enzyme-selective substrates are considered to be in the same order as the mean activities found in human liver microsomes, reveal CYP2A6 to contribute about two times more than the other

CYPs that are active in safrole 1'-hydroxyation [94]. Because CYP2C9, CYP2A6 and CYP2D6 are polymorphic [96], the bioactivation of safrole to 1'-hydroxysafrole in man is expected to be influenced by polymorphisms in these CYPs. Polymorphisms in CYP2C9, CYP2A6 and CYP2D6, leading to poor metabolizer phenotypes, may reduce the relative risk on the harmful effects of safrole, whereas life style factors like the use of alcohol, an inducer of CYP2E1 and barbiturates, inducers of CYP2C9 and polymorphisms in CYP2D6 and CYP2A6 leading to ultra rapid metabolizer phenotypes, may increase the relative risk.

Concluding remarks

As for estragole and methyleugenol, safrole may be converted to a variety of metabolites with different toxicological impact. Identification of the biotransformation enzymes involved in the various bioactivation and detoxification pathways and the implementation of their toxicokinetics into risk assessment models seem to be required for improved methods for extrapolation from high dose animal experiments to low dose carcinogenic risks in man.

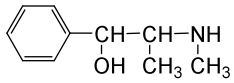
Ephedrine alkaloids

Major characteristics, occurrence and intake

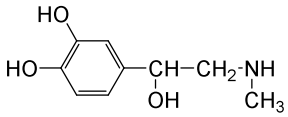
Herbs like *Ephedra sinica*, *Ephedra intermedia* and *Ephedra equisetina*, also known by their Chinese name "Ma Huang", contain so-called ephedrine alkaloids, among which ephedrine (Figure 10) is the dominant one. Other ephedrine alkaloids present include pseudo-ephedrine, nor-ephedrine, methylephedrine, methylpseudo-ephedrine and norpseudo-ephedrine. Certain dietary supplements also include ephedra, with the most popular uses being for improvement of weight loss and athletic performance [97]. Ephedrine, pseudo-ephedrine, and related alkaloids are found in the herb Ma Huang at levels usually up to around 0.5 to 2.5 percent in total [98]. In Ma Huang sold as a powdered herb as well as in standardized extracts, total alkaloid levels may range from 6 to 8 percent, with one product even containing 12 percent [99]. Of the products tested by FDA, the mean contained 21.4 mg per dose [99].

Mechanism of toxic action

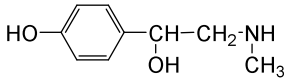
The chemical structure of ephedrine resembles that of the neurotransmitter adrenalin (= epinephrine) (Figure 10). The mechanism of action of ephedrine alkaloids is based on this structural similarity. As adrenalin agonists, these alkaloids produce a sympathomimetic response, characterized by increased heart rhythm, hypertension (elevated blood pressure) and central nervous system stimulation [100-102].



ephedrine



adrenalin (epinephrine)



synephrine

Figure 10 Structure of ephedrine, the major ephedrine alkaloid in ephedra, of adrenalin, the neurotransmitter towards which ephedrine acts an an agonist, and of synephrine, another adrenalin agonist

Doses higher than 50 mg/day/adult may cause heart palpitations, nausea, dizziness, headache, sweating, neuropathy (nerve damage) and tremors. The stimulating effect on the central nervous system can also result in loss of appetite, insomnia, nervousness, seizures and euphoria. At doses above 500 to 1000 mg ephedrine the effects observed are nausea, vomiting, fever, psychoses, spasms, convulsions, respiratory disorders, coma, heart attack and death. A dose of 2000 mg/adult is considered lethal. Chronic exposure to ephedrines may cause behavioral disturbances and psychoses. Several case reports on the toxicity of ephedrine-containing herb preparations have been described, some of them with fatal outcome [97].

Exposure to ephedrine, which is an adrenaline agonist, in combination with drugs known to inhibit monoamine oxidase (MAO) can cause an increased risk on adverse effects. This because the MAO inhibitors block the degradation of adrenalin by MAO, thereby increasing the adrenalin concentration and stimulating the adrenergic neurotransmitter system. Likewise, coexposure with caffeine also has a synergistic effect on the action of ephedrine. Caffeine inhibits phosphodiesterase, the enzyme that hydrolyses cAMP, the second messenger of adrenalin mediated signal transduction. This also stimulates cholinergic neurotransmission.

Polymorphisms of influence

Biodegradation of ephedrine alkaloids may proceed similar to that of adrenalin, by oxidative deamination catalyzed by MAO. Genetic polymorphisms of influence on ephedrine alkaloid toxicity may thus be found at the level of this biodegradation enzyme and/or at the level of the adrenergic receptors for which polymorphisms have been described [103].

Concluding remarks

Recently most countries installed a ban on the use of ephedrine alkaloids in food supplements or other foods. This rule seems to reflect the scientific evidence showing that ephedra poses an unreasonable risk to consumer health.

Synephrine

Major characteristics, occurrence and intake

Synephrine is the main active principle found in the fruit of several Citrus species including *Citrus aurantium* and *Citrus reticulata* [104]. In Traditional Chinese Medicine (TCM) the fruit is also known as Chih-shih. Synephrine occurs in all citrus products in very low concentrations (0.1-2.0%) with 0.25% representing an average value [105]. It is consumed by humans through a citrus fruit containing diet.

Mechanism of toxic action

Synephrine is chemically very similar to ephedrine (Figure 10). Both compounds act on the nervous system in a similar way [106, 107]. Synephrine is a drug in Europe (oxedrine; Sympatol) produced for use as a sympathomimeticum. It acts as a cardiac performance enhancer [108].

Polymorphisms of influence

See remarks made for ephedrine alkaloids.

Concluding remarks

After the FDA banned ephedra, diet-pill companies tried to find a possibly safer alternative turning to synephrine. Whether synephrine may act with potentially fewer side effects, like high blood pressure and increased heart rate, remains to be established. Consumers having high blood pressure or other heart problems should better not use any of these substances.

Kavalactones

Major characteristics, occurrence and intake

The rootstock of the kava (*Piper methysticum*) plant contains a mixture of lipid soluble alpha-pyrones, also called kavapyrones or kavalactones. Pharmacologic activity of kava-kava has been related to six important kavalactones, kawain, 7,8-dihydrokawain, 5,6-dehydrokawain, methysticin, dihydromethysticin and yangonin (Figure 11). High quality kava rhizomes contain 5.5-8.3% kavalactones. Medicinal extracts used in Europe contain 30-70% kavalactones.

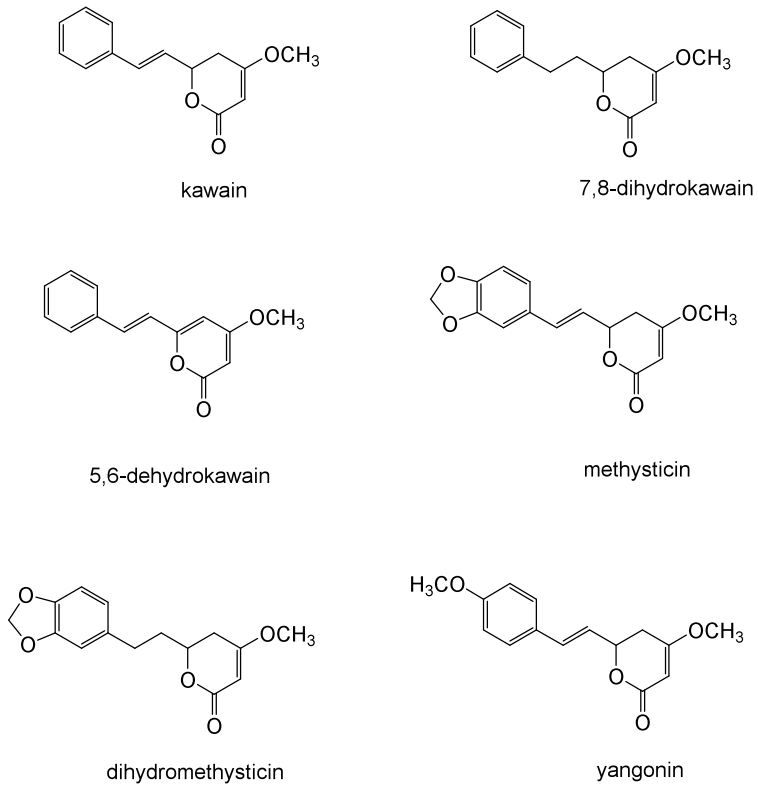


Figure 11 Structures of the major kavalactones from kava-kava

Mechanism of toxic action

Extracts containing high concentrations of kavalactones, at three times 100 mg doses of kava extract standardized to 70% kavalactone content a day, are used for the treatment of anxiety and depression and are claimed to have a calming effect and induce a state of happiness [109, 110]. Through their action on the nervous system they exert sedative, analgesic, anticonvulsant and muscle relaxant effects. The exact mechanism of action for this beneficial effect is not exactly known. Results from animal experiments suggest mechanisms that include inhibition of MAO activity, inhibition of noradrenalin reuptake in the presynaptic neuron and/or action as a dopamine antagonist [111, 112].

The major toxic side effects of kava-kava are dermopathy [113-115] and liver toxicity [116-123]. Since 1999, cases of severe hepatic toxicity in people using kava-containing herbal products have been reported in Europe and the United States [120, 124], including several cases in which patients required liver transplantation following the use of kava-containing products [124]. Liver damage has been reported at intake levels of 60-120 mg kavalactones/day for as short as 14 days. Potential mechanisms underlying the liver toxicity have been related to glutathione depletion or quinone formation [125, 126]. Glutathione was reported to bind irreversibly with kavalactones by a Michael type addition, resulting in opening of the lactone ring (Figure 12A) [125], and high

doses of kavalactones may lead to rapid depletion of GSH followed by toxicity of the lactones to the GSH depleted liver cells [125].

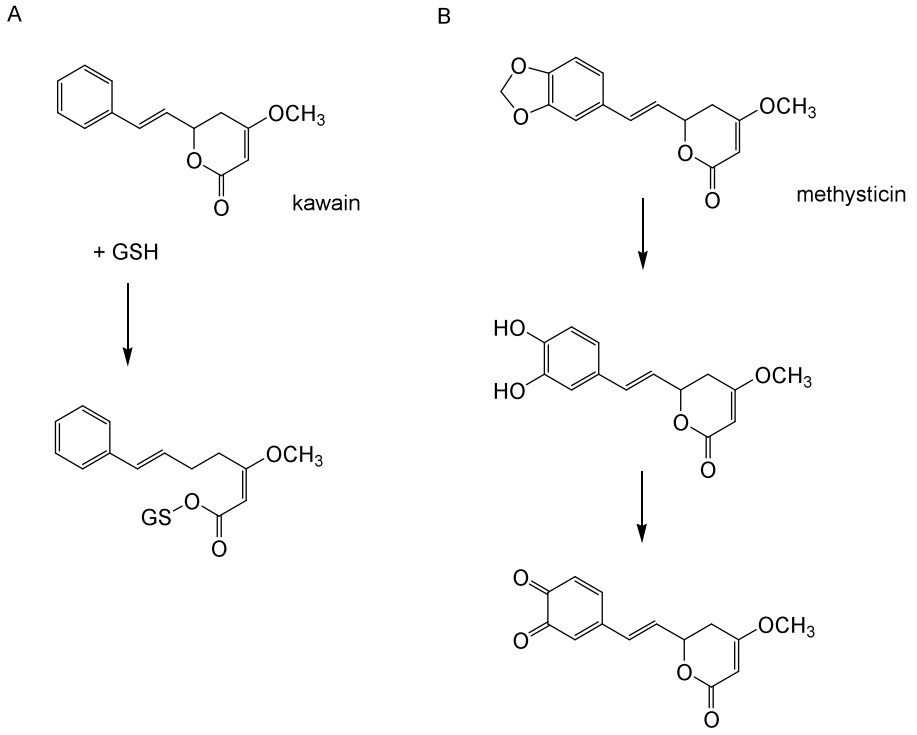


Figure 12 Reactions underlying possible mechanisms for kavalactone induced liver toxicity, including A) glutathione depletion and B) electrophilic quinone formation [125, 126]

For kavalactones containing a methylenedioxyphenyl moiety, such as methysticin and dehydromethysticin, another mechanism may become relevant. CYP catalyzed O-dealkylation of the methylenedioxyphenyl moiety may generate a catechol moiety that may subsequently be oxidized to the corresponding electrophilic o-quinone (Figure 12B) [126]. The CYP isoenzyme(s) catalyzing this bioactivation have not been identified. Alternatively, it has been suggested that kava alkaloids, rather than kavalactones may be responsible for the hepatotoxicty [127].

Polymorphisms of influence

Phenotyping of CYP2D6 activity with debrisoquine in two patients who developed clinical symptoms upon kavalactone ingestion revealed that both were poor metabolizers of debrisoquine. Since the local prevalence of CYP2D6 deficiency was 9%, the probability that two consecutive patients would be deficient was

reported to be less than 0.01% and it was concluded that these data suggest that CYP2D6 deficiency is a risk factor for hepatotoxicity due to kavalactones [128].

Concluding remarks

Several case studies linking kava-kava to liver damage prompted several governments to remove kava-kava extracts from the market. Although it seems generally accepted that kava-kava can be an effective symptomatic treatment option for anxiety [110, 129] the present advices are that these herbal preparations should not be used until the mechanism for hepatotoxicity is clearly ascertained [129].

Anisatin

Major characteristics, occurrence and intake

The spice Chinese star anise (*Illicium verum*) is used in many cultures, mostly for preparing tea. In Southern European countries (France, Spain) star anise is also used against intestinal complaints in children. Recently health problems have been observed due to the use of herb tea containing star anise [130]. In September 2001, in the Netherlands, more than 60 persons showed nausea and vomiting, after drinking a herbal tea called starmix tea, 22 persons were hospitalized due to tonic-clonic insults [131]. EEGs showed epileptiform abnormalities indicating a diffuse cerebral disease [132]. The complaints were ascribed to a toxic star anise species comparable to Japanese star anise (*Illicium anisatum*), which was accidentally exchanged for the non-toxic Chinese staranise (*Illicium verum*) [131]. NMR analysis of this star anise species revealed the presence of anisatin. Anisatin is a sesquiterpenoid (Figure 13) causing numerous other symptoms like lower heartbeat and hallucinations. Because of the latter the Japanese star anise is also called *Illicium religiosum*.

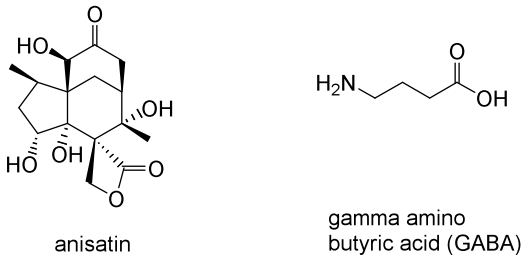


Figure 13 Structure of anisatin and of the neurotransmitter gamma-aminobutyric acid (GABA) for which anisatin is a competitive antagonist

Mechanism of toxic action

Anisatin acts as a non-competitive gamma-amino butyric acid (GABA)-antagonist that can cause tonic-clonic insults [133]. GABA (Figure 13) is the most common message-altering neurotransmitter in the brain and produces stop-signals. It is known that abnormal levels of GABA unbalance the brain's message delivery system

causing a seizure or epileptic attack. Most of the new developments of epilepsy drugs stem from the discovery of GABA.

Polymorphisms of influence

There is no information available.

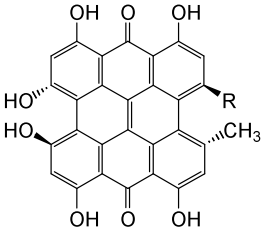
Concluding remarks

Illicium verum has been considered safe for consumption but it contains veranisatins in very low concentrations [134]. Taken the fact that relatively small quantities in infants may be sufficient to produce adverse neurologic reactions and the chances on possible adulteration of *Illicium verum* with *Illicium anisatum,* one could recommend against administering star anise to children [135].

St. John's wort

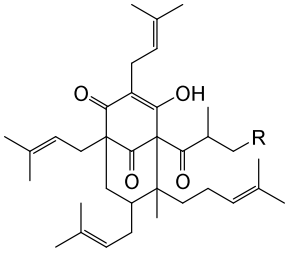
Major characteristics, occurrence and intake

The herbaceous plant St. John's wort (*Hypericum perforatum)* is a member of the *Hypericaceae* family. The fresh plant contains up to 0.3% naphthodianthrones, including up to 0.09% hypericin and 0.23% pseudohypericin (Figure 14). The content of the phloroglucinol compound hyperforin (Figure 14) ranges from 2.0-4.5% of the fresh plant. Adhyperforin (Figure 14), another phloroglucinol compound, comprises 0.2-1.8% of the plant. Phloroglucinols are structurally related to the bitter substances in hops. The phloroglucinols and naphthodianthrones are mainly localized in the flowers and buds. Other constituents include flavonols (e.g. kaempferol, quercetin), flavonoid glycosides (hyperoside, rutin) and biflavonoids (biapigenin) [136-139].



R = CH_3 = hypericin

R = CH_2OH = pseudohypericin



R = H = hyperforin

R = CH_3 = adhyperforin

Figure 14 Structures of the major active ingredients from St. John's wort

St. John's wort is widely used as a treatment for depression. In the United States most St. John's wort is used without medical supervision. Results of a meta-analysis study in 1996 suggested that hydroalcoholic

extracts of St. John's wort were superior to placebo for treatment of mild to moderate depression [140]. Subsequent studies suggested that St. John's wort is comparable to amitryptiline, imipramine, and fluoxetine. However, questions have been raised about methodological limitations of these studies. A double blind, randomized, placebo-controlled trial showed no efficacy of St. John's wort in moderately severe major depression. In this 8-week parallel-group study 340 patients received daily doses of 900-1500 mg of the standardized St. John's wort extract LI-160, 50-100 mg of sertraline or placebo. Neither agent was superior to placebo on the 2 primary outcome measures for which the HAM-D total score and a combination of the HAM-D total score and the CGI score were used. Sertraline performed better than placebo on the secondary measure CGI- improvement scale [141]. Several studies indicate that St. John's wort extracts inhibit synaptosomal uptake of serotonin, dopamine and noradrenalin. Results of an in vitro study suggested that hyperforin is responsible for the inhibition of serotonin uptake [142].

Mechanism of toxic action

Side effects of treatment with St. John's wort tend to be mild and include swelling, anorgasmia and frequent urination [141]. Several cases of increased sensitivity to sunlight following use of St. John's wort were reported. These reactions however are rare and may be encountered following prolonged exposure at high or very high doses [139]. In a study on the antiviral effects of hypericin, in patients with chronic hepatitis C virus infections, 5 of 12 subjects receiving 0.05 mg/kg bw/day orally for 8 weeks and 6 of 7 subjects receiving 0.10 mg/kg bw/day developed phototoxic reactions. In both groups paresthesias were the most common reactions followed by dermatitis. In the high dose group darkened coloration and pruritic nodules were also reported. Apart from these effects no other serious adverse events were reported [143]. Similar phototoxic reactions were found in a study when intravenous hypericin was given to HIV-infected patients [144]. One could hypothesize that individuals with certain virus infections are more prone to develop phototoxic reactions to hypericin.

Of concern is the capability of St. John's wort to interact with certain drugs. In 1999, a review of available literature published in the Lancet [145] raised questions regarding the safety of St. John's wort when used concomitantly with various prescribed drugs. This review discussed 8 cases of interactions between St. John's wort and concomitant medications. The drugs for which interactions of St. John's wort are of concern are drugs that are metabolized by hepatic CYP enzymes. This is because ingredients in St. John's wort induce specific CYP activities involved in drug inactivation. Because of CYP induction plasma concentrations of certain drugs can become subtherapeutic [145]. Subsequently, the European Agency for the Evaluation of Medicinal products issued a warning for this potential effect of St. John's wort [146].

Polymorphisms of influence

Recently, the effect of St. John's wort on the activity of different CYP2C19 genotypes was studied by investigating mephenytoin pharmacokinetics in 12 healthy subjects by administering a single dose of the drug after two weeks of St. John's wort treatment [147]. Mephenytoin is almost exclusively metabolized by CYP2C19. Treatment with St. John's wort significantly increased mephenytoin metabolism in 6 wild-genotype CYP2C19*1/*1

subjects. The other 6 subjects which were homozygous for CYP2C19*2 or heterozygous for CYP2C19*2/*3 were found to be poor metabolizers of mephenytoin. In the poor metabolizers, St. John's wort exerted no significant effect on mephenytoin metabolism. St. John's wort administration did not significantly alter CYP1A2 activity determined by measuring caffeine metabolism [147]. When the effect of St. John's wort on the metabolism of amitriptyline and its metabolites was studied no correlation between CYP2D6, 2C9 or 2C19 genotype and amitriptyline pharmacokinetics could be observed [148].

In humans, one case of reduced plasma concentrations of theophylline, a drug metabolized by CYP1A2 and combined use of St. Johns wort was reported. However, a study of 12 healthy subjects taking a single dose of theophylline after two weeks of St. John's wort use failed to show significant changes in theophylline plasma concentrations compared to theophylline plasma concentrations without treatment with St. John's wort [149]. CYP 3A4 and CYP 3A5, members of the CYP 3A subfamily, are the most abundantly expressed CYP enzymes in the liver and gastrointestinal tract of humans. They metabolize more than 120 frequently prescribed drugs [150]. A study in mice showed that St. John's wort induced the CYP3A subfamily and that hyperforin played an important role in this effect [151]. When human hepatocytes were treated *in vitro* with hyperforin and St. John's wort, expression of CYP3A4 was markedly increased by either treatment. Hyperforin accounted for much of the effect of St. John's wort in this study [152]. By contrast, treatment of rats for 10 days with St. John's wort failed to show an increased expression of hepatic CYP3A. Amounts of CYP1A2, multidrug resistance protein 2 (MRP2) and glutathione S-transferase-pi (GST-pi) however were increased up to respectively 357%, 304% and 252% of controls in the liver of rats exposed for 10 days to 400 mg St. John's wort suspension/kg bw/day estimated to be an antidepressant effective dose in rats [153].

A systemic review of clinical trials concerning interactions of St. John's wort with prescribed drugs revealed that of 19 trials for which drug plasma data were available, 17 found a decrease in the systemic bioavailability of the drug upon coadministration with St. John's wort [154]. Cotreatment with St. John's wort was shown to reduce plasma concentrations of the antineoplastic agent irinotecan by 42% [155]. In healthy volunteers St. John's wort reduced the area under the curve in plasma of the HIV-1 protease inhibitor indinavir by 57% [156]. Two cases of acute rejection in heart transplant patients were reported where St. John's wort was used concomitantly with the immunosuppressive agent cyclosporin [157]. Other drugs affected by St. John's wort are the tricyclic antidepressants amitriptyline and it's metabolite nortriptyline, digoxin, ethinylestradiol and warfarin [145, 148, 158-160]. Table 1 presents an overview of examples of interactions of St. John's wort with drugs [145, 147, 148, 155-165]. There have been reports on pharmacodynamic interactions of St. John's wort with drugs. Combined use of St. John's wort with serotonin reuptake inhibitors, antidepressant drugs by 5 elderly patients resulted in symptoms characteristic of central serotonin excess. These symptoms include changes in mental status, tremor, gastrointestinal upset, headache, myalgia and restlessness [145].

Concluding remarks

Evidence of interactions of St. John's wort with an increasing number of commonly used medicines is growing and more attention is required to prevent adverse effects of concomitant use of St. John's wort and

drugs. When used properly this drug can be used safely. Medical staff should routinely ask patients for self-medication with St. John's wort and other herbs. Governmental bodies need to focus on prevention measures including communication of risks of interactions of certain food supplements with medicines to the general public.

Table 1 Examples of interactions of St. John's wort with drugs

Drug	Drug type	Result of interaction	Possible mechanism	Ref.
Cyclosporine	Immunosuppressant	Reduced plasma concentration	Induction of CYP3A4 or P-glycoprotein	[157,158, 161]
Tacrolimus	Immunosuppressant	Decreased tacrolimus AUC	Induction of CYP3A or P-glycoprotein	[162]
Digoxin	Cardiovascular drug	Reduction of digoxin AUC	Induction of CYP3A or P-glycoprotein	[160]
Ethinyl oestradiol/ dienogesterol and other contraceptives	Oral contraceptive	Unexpected pregnancy, intermenstrual bleedings	Induction of CYP3A4	[159, 163]
Indinavir	HIV-1 protease inhibitor	Reduced plasma concentration	Induction of CYP3A4	[156]
Irinotecan	Antineoplastic agent	Reduced plasma concentration	Induction of CYP3A4	[155]
Mephenytoin	Anticonvulsant	Increased urine clearance of metabolite	Induction of CYP2C19	[147]
Warfarin	Anticoagulant	Reduced anticoagulant effect	Induction of CYP2C9	[159]
Amitriptyline, Nortriptyline	Tricyclic antidepressants	Reduced plasma concentration	Induction of cytochrome P-450, mainly CYP3A4 or P-glycoprotein	[148]
Buspirone	Anti anxiety drug	Serotonin syndrome	Overstimulation of 5-HT receptors	[164]
Nefazodon, Sertraline	Selective serotonin reuptake inhibitor antidepressants	Manic episodes, central serotonin excess	Overstimulation of 5-HT receptors	[145, 165]

Cyanogenic glycosides

Major characteristics, occurrence and intake

Cyanogenic glycosides are present in a number of food plants and seeds and include compounds like amygdalin, dhurrin, linamarin, linustatin, lotaustralin, neolinustatin, prunasin, sambunigrin and toxiphyllin. Figure 15 presents some relevant cyanogenic glycosides including linamarin, present in the roots of cassava (*Manihot esculenta*) at levels of 10-1120 mg HCN (free and bound)/kg, as well as in Lima bean seeds (*Phaseolus lunatus*)

at levels of 100-3000 mg HCN (free and bound)/kg, dhurrin from sorghum, and amygdalin, a natural substance found in seeds of apples and pears, as well as in the leaves, fruit and seeds of black cherry, almond, cherry, plum, peach and apricot trees at levels that may be as high as 300-4000 mg HCN (free and bound)/kg [166]. Holzbecher et al. [167] report that apricot seeds contain 2.92 mg/g HCN and peach seeds contain 2.60 mg/g HCN, while apple seeds contain only 0.61 mg/g HCN. The mean and 97th percentile overall daily intake of HCN have been estimated to be about 46-95 and 214-372 µg/person which corresponds to 0.7-1.4 and 3.3-5.4 µg/kg bw/day [166]. Food products containing relatively high levels of HCN (free and bound) are almonds and/or marzipan containing confectionery and baked goods, that may contain levels up to 40 mg/kg, with raw marzipan paste containing the highest level of 50 mg HCN (free and bound)/kg [166].

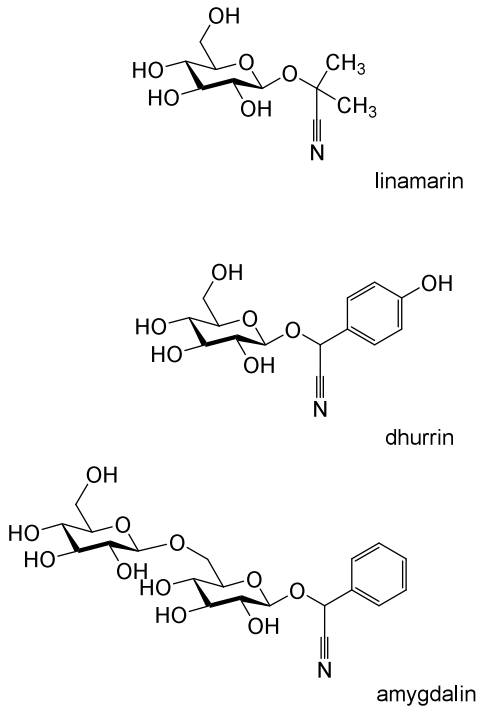


Figure 15 Examples of three important cyanogenic glycosides, linamarin from cassava, dhurrin from sorghum and amygdalin from, for example, apricot kernels and apple kernels

Mechanism of toxic action

Cyanogenic glycosides are a cause of concern, because once ingested they are metabolized to hydrogen cyanide (HCN). Hydrogen cyanide is released from the cyanogenic glycosides by plant beta-glucosidases, which come into contact with the cyanogenic glycosides when fresh plant material is macerated as in chewing, or by beta-glucosidases present in the gut flora. Figure 16A presents the hydrolysis of amygdalin to HCN in the gastrointestinal tract. It is a two-step process catalyzed by the enzymes beta-glucosidase (produced

by intestinal bacteria) and hydroxynitrile lyase [168]. The cyanogenic glycosides, linamarin and lotaustralin from cassava are converted to HCN in the presence of linamarase, a naturally occurring enzyme in cassava. Linamarase acts on the glycosides when the cells are ruptured. Cassava is an important source of carbohydrate for people in Africa and South America. The toxic ingredients are detoxified by hydrolysis through chopping and grinding in running water prior to preparation [169].

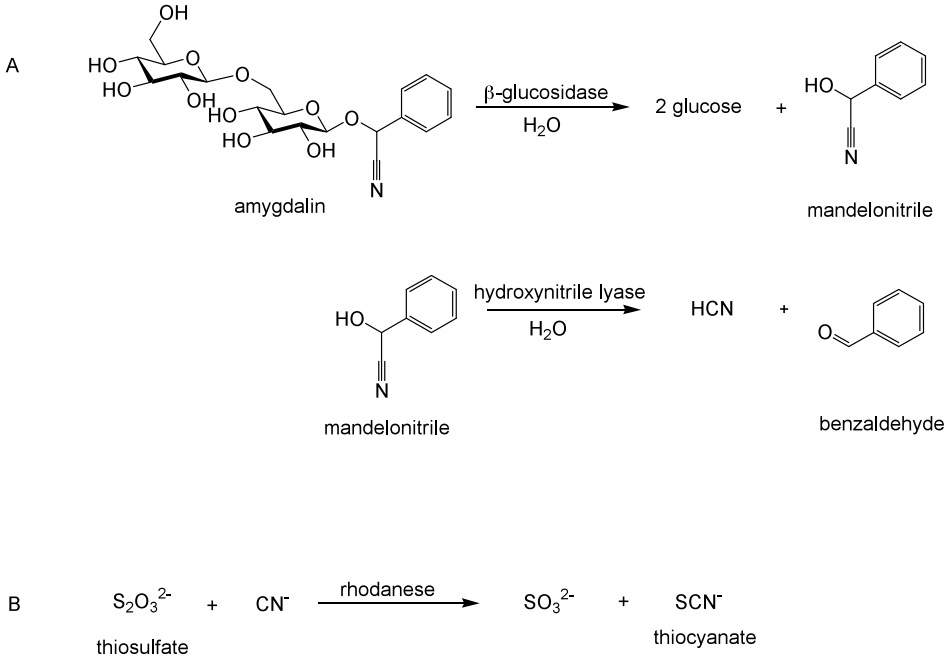


Figure 16 Enzymatic conversion of amygdalin to cyanide (A), and conversion of cyanide by rhodenese generating thiocyanate (B)

Cyanide causes toxic effects by binding to cytochrome oxidase the terminal enzyme in the mitochondrial electron transport chain. By hampering the generation of ATP and oxygen utilization, a histotoxic anoxia is produced. In small doses, the body can detoxify cyanide. In man, cyanide (CN^-) is detoxified by conversion in the liver to thiocyanate by rhodanese (Figure 16B), by direct chemical combination with sulphur containing amino acids or by combination with hydroxycobalamine. Methaemoglobin effectively competes with cytochrome oxidase for cyanide and the formation of methaemoglobin from hemoglobin by therapeutically added nitrite or amylnitrite is used in the treatment of cyanide poisoning. If untreated, large doses of cyanide are fatal [170-173]. The acute lethal oral dose of cyanide for humans is reported to vary between 0.5 to 3.5 mg CN^-/kg bw [171-173] and the sensitivity to cyanide may be highly variable depending on age, body mass and health status of the individual [174].

Holzbecher et al. [167] report that apricot seeds contain 2.92 mg/g HCN and peach seeds contain 2.60 mg/g HCN, while apple seeds contain only 0.61 mg/g HCN. Accordingly, these authors conclude that a person can easily consume a lethal quantity of apricot or peach seeds, but is unlikely to eat a lethal amount of apple

seeds. The consumption of 60 bitter almonds, containing an average cyanide content of 6.2 mg HCN/ bitter almond [175] (leading to an intake of 6.2 mg HCN/kg bw which is above the acute lethal oral dose of 0.5 –3.5 mg/kg bw), has been reported to be deadly for an adult [176]. Several case studies with fatal outcome upon ingestion of high levels of amygdalin have been reported [175, 177-179]. Acute cyanide toxicity at small doses can cause headache, tightness in throat and chest, and muscle weakness.

The effects of chronic (long-term) exposure to cyanide are less well known. Chronic exposure to linamarin from cassavas, has been reported to cause malnutrition, diabetes, congenital malformations, neurological disorder and myelopathy [180, 181]. It has been proposed as the cause of epidemics of Konzo, a form of tropical myelinopathy with sudden onset of spastic paralysis [182, 183]. Degeneration of the corticospinal motor pathway in affected individuals may be the result of the production of thiocyanate from linamarin and the stimulation of neuronal glutamate receptors by thiocyanate [25, 184]. Thiocyanate is formed from HCN by the mitochondrial enzyme rhodanese (Figure 16B), a reaction that is generally considered a detoxification pathway of cyanide. The less toxic thiocyanate is excreted in the urine [176, 185]. In countries with low iodine uptake, thiocyanate exposure after cassava consumption might be a risk factor for goiter [186]. Goiter is thought to occur when cyanogenic glycosides are present at a level of 10-50 mg/kg in food. This thyrotoxic effect of cyanide results from the action of thiocyanate as an iodine antagonist. In the 1970's and early 1980's, amygdalin was proposed as an anticancer drug (also named laetrile and vitamin B17). However, the dangers and ineffectiveness of laetrile were soon recognized. The American Cancer Society has since then indicated that laetrile is a 'toxic drug that is not effective as a cancer treatment'. And in recent years products containing amygdalin, including apricot kernels, were banned as over-the-counter products in many countries.

Polymorphisms of influence

There is no information available.

Concluding remarks

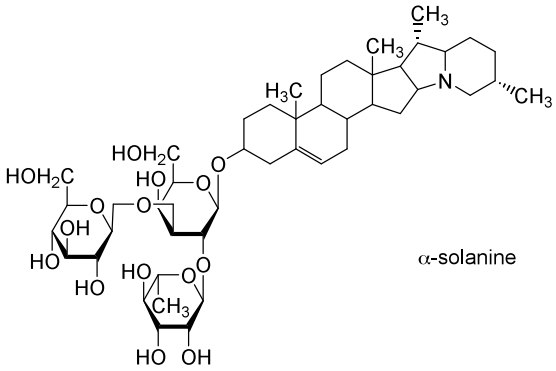
Several regulatory agencies have evaluated the toxicological and epidemiological data in order to establish a safe level of intake of cyanogenic glycosides. A numerical value for safe intake levels, i.e. a TDI, has not been derived, and it is generally concluded that application of limits for the presence of HCN in foods and beverages should be continued.

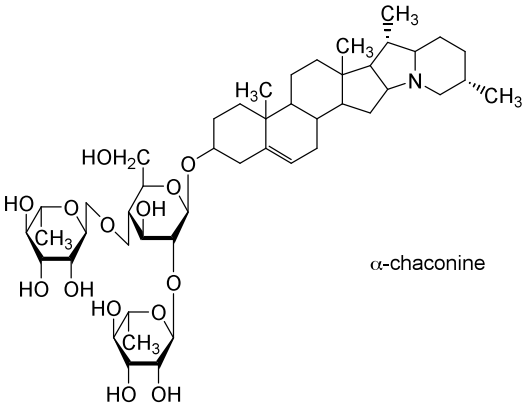
Solanine and chaconine

Major characteristics, occurrence and intake

Alpha-solanine and alpha-chaconine (Figure 17) are toxic saponin-like alkaloids. They exist as beta-D-glycosides, and are present in potatoes (*Solanum tuberosum*). The total glycoalkaloid content in potatoes normally varies between 2-10 mg/100 g and in chips between 2 and 60 mg/100 g, the latter due to the fact that

processing of the potato in order to make chips may increase the amounts of glycoalkaloids [187]. FDA regulations limit the solanine content in potatoes to no more than 20 mg/100 g.





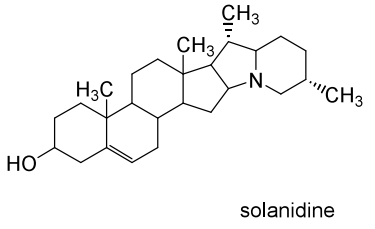


Figure 17 Structures of alpha-solanine, alpha-chaconine and solanidine from potatoes

Mechanism of toxic action

The compounds inhibit cholinesterase enzymes: butyrylcholinesterase (BuChE), that is concentrated in the liver and lungs, and acetylcholinesterase (AChE), that is required to hydrolyze and inactivate the neurotransmitter acetylcholine [188, 189]. The mechanism of this inhibition was shown to be reversible and at a

concentration of 2.88 x10^{-5} M alpha-chaconine and alpha-solanine were shown to inhibit BuChE by about 70% and 50% respectively [190]. Changes in the glycoalkaloid content of potatoes may occur during storage, under the influence of light and radiation, following mechanical damage and as a result of food processing. Solanine is heat stable and insoluble in water; therefore cooking does not remove the toxicant. Human toxicity from ingestion of green potatoes with a high solanum glycoalkaloid content is associated with gastric pain, weakness, nausea and vomiting. The potential for teratogenic effects has been a significant public concern in populations consuming large amounts of potatoes. The concern arises from studies with Syrian hamsters. Animals treated orally with potato sprouts containing solanidine (Figure 17), the common aglycon of alpha-solanine and alpha-chaconine, had offspring with craniofacial malformations [191]. A 1972 report of Renwick [192] suggesting certain birth (neural tube) defects in humans in areas with higher consumption of potatoes infested with Phytophthora infestans, an infectious potato disease inducing the amount of solanine, could not be supported in animal experimental and human studies [193, 194]. The 2-week and 90-day NOAEL's (No Observed Adverse Effect levels) for solanine are 35 and 22.5 mg/kg bw respectively.

Polymorphisms of influence

There is no information available.

Concluding remarks

There is a long history of human consumption of plants containing glycoalkaloids, and the consumption of potatoes with normal glycoalkaloid levels found in properly grown and handled tubers are not of concern.

Thujone

Major characteristics, occurrence and intake

The terpenoids alpha- and beta-thujone (Figure 18) occur together in the essential oils and parts of the plants of *Artemisia absinthium* (wormwood), *Salvia officinalis* (sage), *Salvia sclarea* (clary), *Tanacetum vulgare* (tansy) and in *Juniperus* spp. The ratio of alpha- to beta-thujone varies with the source [195]. Synthetic alpha-thujone is also available commercially. Thujone is present in food ingredients with flavoring properties. Estimates of intakes of thujone have been made in France and the United Kingdom. In France, the mean and the 97.5th percentile daily intakes were estimated to be 15.6 and 44.3 µg/kg bw/day respectively. The intakes in the United Kingdom were estimated to be somewhat lower at 3.9 and 14.2 µg/kg bw/day respectively. The major dietary contribution to intake appeared to derive from sage and sage-flavored products and alcoholic beverages including absinthe [195]. Absinthe is an emerald-green liquor that was very popular at the end of the 19th century. It was associated with the Bohemian lifestyle and was coupled to the inspiration of famous artists and poets. Because of its widespread abuse and the associated toxicity of its content of oil of wormwood, absinthe was made illegal in most countries in the 1910s.

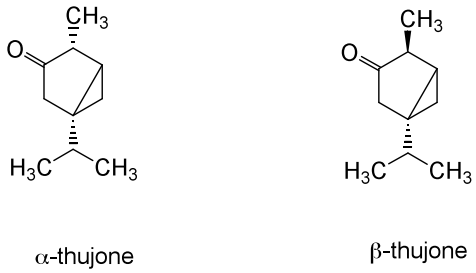


Figure 18 The active principles from absinthe; alpha- and beta-thujone

Mechanism of toxic action

The most likely ingredient responsible for the toxicity is believed to be alpha-thujone. The content of beta-thujone often exceeds that of alpha-thujone, but the beta-stereo isomer is generally considered to be of lower toxicity than alpha-thujone [195-197]. The thujone content of old absinthe was about 260 ppm. Already by the end of the 19th century it was recognized that absinthe could cause convulsions, hyperactivity, excitability, hallucinations, and psychotic behavior, including suicide [196]. Other reported side effects of thujone and wormwood are nausea, vomiting, insomnia, restlessness, vertigo, tremors and seizures. Large doses of thujone have been found to cause delirium, convulsions, seizures, paralysis, brain damage, renal failure and death.

The mechanism of neurotoxicity of alpha-thujone has been ascribed to the fact that it blocks the receptors for gamma-aminobutyric acid (GABA) in the brain [196]. Without access to GABA, a natural inhibitor of nerve impulses, neurons fire too easily and their signaling goes out of control. Thus, the mechanism underlying the adverse effect of alpha-thujone is comparable to that of anisatin (section 2.9).

Polymorphisms of influence

There is no information available.

Concluding remarks

In the 1990s absinthe has become popular again. Its newly fashionable image, combined with possibilities for global purchase through the Internet has helped initiate its revival. The currently available versions of absinthe have levels of thujone of about 10 ppm. This is below the current upper limit set in Annex II of Directive 88/388 EEC of 35 mg/kg (ppm) in bitters. The EU-SCF considered the available data inadequate to establish a TDI (Tolerable Daily Intake) but noted that some of the deficiencies in the database were being addressed in ongoing studies and they recommended that the results of these should be reviewed when available [195]. Current levels of alpha- and beta-thujone in absinthe are however judged to be of less toxicological concern than its ethanol content [198].

Glycyrrhizinic acid

Major characteristics, occurrence and intake

Glycyrrhizinic acid (Figure 19) is a food flavoring substance extracted from the roots and rhizomes of the liquorice plant (*Glycyrrhiza glabra*). Because of its sweetness this triterpenoid saponin compound (33-200 times as sweet as sucrose) is an important component of a range of foodstuffs such as liquorice confectionery, tooth paste, cough drops, herbal teas, chewing gum, chewing tobacco and several alcoholic beverages (e.g. pastis). Herbal liquorice teas can contribute significantly to the intake of glycyrrhizinic acid. A Dutch study found for prepared herbal liquorice teas a mean concentration of 149 mg/l glycyrrhizinic acid (range 25-450 mg/l). The mean concentration of glycyrrhizinic acid in liquorice was 0.15% [199].

In the Netherlands, liquorice confectionery is consumed in relatively high quantities. In 1998, 0.4% of the Dutch population consumed 50 g of liquorice per day and 0.1% consumed more than 100 g per day leading to intakes of glycyrrhizinic acid of more than 75 mg and 150 mg per day, respectively [200]. The average daily consumption among regular consumers was 11.5 g/person [201]. In herbal medicine the root is used as Liquiritiae radix for treating cough, inflammation of the upper respiratory tract, gastritis and gastric ulcers. Glycyrrhizinic acid is present in the liquorice root as ammonium and calcium salts. When hydrolyzed glycyrrhizinic acid yields di-glucuronic acid and glycyrrhetic acid (Figure 19). Other constituents of liquorice root are triterpenoid saponins (e.g. 24-hydroxyglycyrrhizin, glabranin A and B), flavonoids (e.g. the isoflavan glabridin) and coumarins [202].

Mechanism of toxic action

An essential step for absorption in the gastro-intestinal tract is the hydrolysis of glycyrrhizinic acid by intestinal bacteria into glycyrrhetic acid, which is the ultimate biologically active molecule (Figure 19).

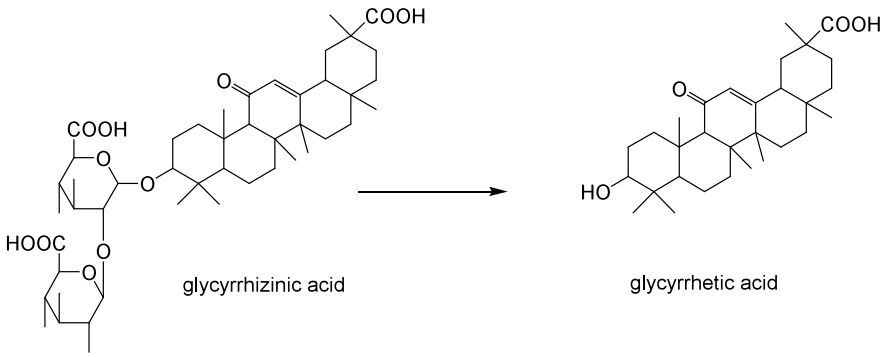


Figure 19 Structural formula of glycyrrhizinic acid and its conversion to the biologically active metabolite glycyrrhetic acid

Absorption of glycyrrhyzinic acid, in the form of glycyrrhetic acid, from solutions or from liquorice is comparable and virtually complete. Due to its lipophilic nature excretion of glycyrrhetic acid by the kidney is very low. After a slow uptake by the liver the substance is subject to entero-hepatic circulation. Glycyrrhetic acid is the

biologically active metabolite, which inhibits the enzyme 11-beta-hydroxysteroid dehydrogenase-2 (11-BOHD-2). This enzyme is found in the distal kidney tubules and converts the steroid hormone cortisol to cortisone. Cortisol binds to the mineralocorticoid receptor but cortisone does not. A decreased activity of 11-BOHD-2 leads to an excess of cortisol and an overstimulation of this mineralocorticoid receptor. This causes water and sodium retention and an increased excretion of potassium. When exposed to large doses of glycyrrhizinic acid over a prolonged period, this electrolyte imbalance and the water retention can cause hypokalaemia, hypernatraemia, edema, hypertension and cardiac disorders [201, 203].

Polymorphisms of influence

An *in vitro* study showed that liquorice root extract and its major flavonoid glabridin inhibited human CYP3A4. Furthermore, glabridin inhibited CYP2B6, which is responsible for the metabolism of roughly 3% of prescribed drugs such as ketamine, phenobarbital and rifampin [204]. Glycyrrhizinic acid was found to increase plasma concentrations of prednisolone in humans [205]. In a repeated dose-response study in human volunteers a NOAEL of 2 mg/kg bw/day glycyrrhizinic acid was derived.

Concluding remarks

Because of limited data the EU-SCF [201] could not derive an ADI for glycyrrhizinic acid. The Committee was however of the opinion that ingestion of glycyrrhizinic acid should not exceed 100 mg/day which should protect the majority of the population. Yet certain subgroups with a decreased 11-BOHD-2 activity or hypertension might not be sufficiently protected [201].

Conclusions

The present review describes the nature and mechanism of action of the phytochemicals nowadays receiving increased attention in the field of plant and herb-based food items, and Table 2 gives an overview of the data presented. From this it can be concluded that a variety of phytotoxins may cause concern, because of their bioactivation to reactive alkylating intermediates that are able to react with cellular macromolcules causing cellular toxicity, and, upon their reaction with DNA, genotoxicity resulting in tumors. Another group of phytotoxins of present concern is active without the requirement for bioactivation and, in most cases, appear to act as neurotoxins interacting with one of the neurotransmitter systems.

For most compounds regulatory agents are aware of the problems encountered and have taken or are considering appropriate regulatory actions to protect the public. These regulatory actions may vary from setting TDIs (such as for example for coumarin), application of limits for the presence of a compound in foods and beverages (such as for HCN, thujone and glycoalkaloids), trying to define safe upper limits (such as for beta-carotene), advising on a strategy aiming at restrictions in use (such as for estragole, methyleugenol and safrole), informing the public to be cautious and aware of possible adverse side effects, (as for St. John's wort, glycyrrhizinic acid and kava-kava), or taking specific plant varieties and/or their ingredients from the market (such

as for aristolochic acids, pyrrolizidine alkaloids and kava-kava). In spite of this regulatory awareness, and previous and recent regulatory actions taken, it cannot be excluded that specific developments may still result in problems. This includes i) phenomena such as overconsumption by particular groups, sometimes stimulated by companies making illegal claims on their websites or in their literature, ii) the fact that many consumers equate "natural" with "safe" when considering plant-based food supplements or drug preparations, iii) the over-the-counter selling of food supplements through Internet sites from countries where regulations are not in place, and iv) the fact that there is yet no system in place to guarantee the safety and quality of botanical supplements.

Table 2 Overview of the food-borne phytochemicals of present concern in food toxicology and the mechanism of their toxic effect as discussed in the present paper

Compound(s)	Requires bioactivation	Mechanism of action
Aristolochic acids	+	Formation of reactive nitrenium ion causing Chinese Herb Nephropathy and urothelial cancers
Pyrrolizidine alkaloids	+	Formation of pyrrolic dehydro-alkaloid metabolites alkylating DNA and other macromolcules, causing liver cell necrosis and liver cancer
Beta-carotene	+	Oxidation products resemble retinal, disturbing retinal homeostasis leading, in combination with cigarette smoke, to (lung) tumor promotion
Coumarin	+	Formation of toxic tumor inducing metabolites in the coumarin-3,4-oxide pathway
Alkenylbenzenes: safrole, methyleugenol, estragole	+	Formation of genotoxic, carcinogenic 1'- sulfooxymetabolites
Ephedrine alkaloids	-	Adrenalin agonist
Synephrine	-	Adrenalin agonist
Kavalactones	+/-	Glutathione depletion and/or quinone formation
Anisatin	-	GABA antagonist
St. John's wort ingredients	-	Interfere with CYP-mediated biotransformation resulting in drug interactions
Cyanogenic glycosides	+	Release of cyanide, inhibits cytochrome c oxidase
Solanine	-	Cholinesterase inhibition
Thujone	-	GABA antagonist
Glycyrrhizinic acid	+	Hydrolysis generates glycyrrhetic acid that inhibits 11-beta-hydroxysteroid dehydrogenase-2 (11-BOHD-2), leading to an excess cortisol and overstimulation of the mineralocorticoid receptor, causing water and sodium retention and increased potassium excretion

The latter is especially worrying as products on the market are known be of variable quality with high variation in the content of the active but also of the toxic principles, and the fact that already several examples of replacement of a harmless variety with a toxic alternative have occurred, either intentionally or accidentally. Misidentification of

plants harvested from the wild may add to the problem. The growing volume of products and sales call for a more formal pre-marketing assessment and better and stricter controls than presently available.

Altogether the examples above illustrate that “natural” does not equal “safe” and that in modern society adverse health effects, upon either acute or chronic exposure to phytochemicals, can occur as a result of (mis)use of plant- or herb-based foods, botanicals or botanical preparations intended for human consumption as food supplements, teas or other extracts. At present regulatory bodies have become more aware of the problem and are increasing their efforts to ensure the safety of botanical supplements [206, 207].

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CHAPTER
4

Based on:

Martijn J Martena, Jacqueline CA van der Wielen,
Leo FJ van de Laak, Erik JM Konings, Henk N de Groot
and Ivonne MCM Rietjens

Enforcement of the ban on Aristolochic acids in Chinese traditional herbal preparations on the Dutch market

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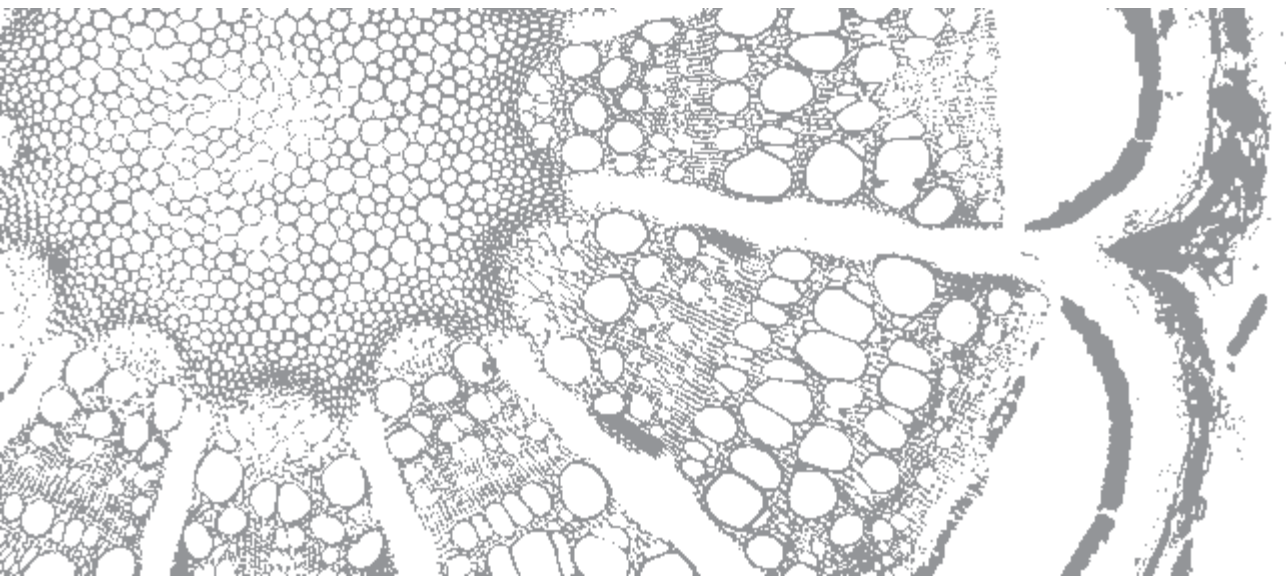
Determination of aristolochic acids in Chinese traditional herbal preparations on the Dutch market



Abstract

In Traditional Chinese Medicine (TCM) several *Aristolochia* species are used. *Aristolochia* spp. contain a mixture of aristolochic acids (AAs) mainly AA I and AA II, which are nephrotoxicants and carcinogens. After AA related nephropathy (AAN) and urothelial cancer were described in female patients in Belgium following intake of AA contaminated herbal preparations, herbs with AAs were prohibited worldwide. Confusing nomenclature can cause AA contamination of certain Chinese traditional herbal preparations (THPs). Here we report the results of investigations by the Dutch Food and Consumer Product Safety Authority (VWA) into the presence of AAs in THPs sampled on the Dutch market using an LC-MS method. Between 2002 and 2006 we sampled 190 Chinese THPs using recent information on Chinese THPs potentially containing AAs. AA I was found in 25 samples up to a concentration of 1676 mg/kg. AA II was also found in 13 of these samples up to 444 mg/kg. All 25 positive samples including Mu Tong, Fang Ji, Tian Xian Teng and Xi Xin were part of a group of 68 THPs identified as possibly containing AAs. In a worst-case scenario use of a sample of Mu Tong with the highest AA content over a 7-day period would result in the same intake levels of AAs, which significantly raised the cancer risk in the Belgian AAN cases. Our results show that contaminated THPs still can be found on the market following worldwide publicity. Therefore it can be concluded that testing of possibly AA contaminated THPs is still essential.

Introduction

In Traditional Chinese Medicine (TCM) *Aristolochia* species such as *A. fangchi* and *A. manshuriensis* and others are used to treat snake and insect bites, promote lactation or urination and reduce edema [1]. *Aristolochia* spp. are used for medicinal purposes worldwide. Many herbs from the genus *Aristolochia* and several species of the genus *Asarum*, both belonging to the family of the *Aristolochaceae*, contain several aristolochic acids (AAs) often accompanied by aristolactams [2-4]. Literature on the toxicity of the *Aristolochiaceae* and related analytical papers mostly focus on a naturally occurring mixture of AAs mainly consisting of aristolochic acid I (AA I) and aristolochic acid (AA II) (Figure 1) [5].

AAs were shown to be nephrotoxic and carcinogenic in animal studies with rodents [5,6]. It has been shown in rat studies that the renal proximal tubule is an important target of AA toxicity, which can result in renal failure [7,8]. AAs have been suggested to play a role in the Balkan endemic nephropathy (BEN) characterized by renal interstitial fibrosis. Seeds of *Aristolochia clematitis,* which is endemic to BEN affected areas, may have contaminated grain [9]. The International Agency for Research on Cancer (IARC) concluded that herbal preparations with *Aristolochia* spp. are carcinogenic to humans and that naturally occurring mixtures of AAs probably are human carcinogens as well [10]. In humans, the hepatic and renal activation of AAs is attributed to reductive metabolic activation by cytochrome P450 (CYP) 1A1, CYP1A2, NAD(P)H:quinone oxidoreductase and others. The resulting ultimate carcinogenic species is able to form adducts with DNA which in turn can cause mutations and neoplastic changes [5, 11]. We recently reviewed the toxic action of AA in some detail [12].

TCM is gaining popularity in Western countries but certain safety issues of Chinese traditional herbal preparations (THPs) such as the deliberate use of high amounts of heavy metals [13] and the presence of AAs invariably require attention. In the US and the Netherlands Chinese THPs are regarded as foods. In Dutch food law THPs are regulated as herbal preparations in the Commodities Act Decree 'Herbal preparations'. Since it came into force early 2001, this Decree has prohibited the presence of AAs and their derivatives in herbal preparations with *Aristolochia* spp. This ban was recently extended to all herbal preparations irrespective of the plant species present. Several other countries such as the UK, the USA, Canada, Australia and New Zeeland have since 2000 significantly limited or prohibited the sale of AA containing herbs and issued warnings [14-18]. These measures were inspired by a steadily expanding insight into the nature of the causative agent of poisonings with Chinese THPs in Belgium in the early 1990s.

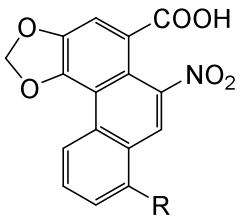


Figure 1 Structures of AA I (R = OCH_3) and AA II (R = H)

In 1992, a cluster of 9 similar cases of renal interstitial fibrosis in female patients was identified in Brussels. All these patients were treated between 1990 and 1992 in a slimming clinic with a regimen consisting of a diet, injections and capsules containing pharmaceuticals such as fenfluramine, herbal preparations and a pancreas extract. Early 1990, the clinic had altered the THP formulation by introducing the Chinese herbs *Magnolia officinalis* and *Stephania tetrandra* to the capsules replacing other herbs. It was suggested that *S. tetrandra* was inadvertently replaced by *Aristolochia fangchi*. TLC detection of AAs in these herbal preparations failed however [19]. Afterwards, AAs were found in 11 of the 12 batches of *S. tetrandra* powder delivered to Belgian pharmacies during the treatment period [20]. The disease became known as Chinese herb nephropathy (CHN). After the initial report, more than 100 cases of rapidly progressing renal fibrosis associated with exposure to AAs were identified in Belgium and approximately 170 cases of AA associated CHN were described in other European countries, the USA and in Asia [5, and references therein].

In renal tissue of 39 patients who were treated with the Belgian slimming regimen and who were followed for CHN related end stage renal failure, 18 cases of urothelial cancer were identified. All examined tissue samples contained AA related DNA adducts [21]. It was found that a total intake of more than 200 g *S. tetrandra* (probably mostly replaced by *A. fangchi*) was associated with a higher risk of urothelial carcinoma [21]. A statistical analysis of the prescriptions and medical files of 71 CHN patients showed that of all administered drugs only the cumulative dose of the contaminated *S. tetrandra* preparation could predict the renal failure progression rate [22]. The typical chronic interstitial lesions of CHN were reproduced in a rats injected with 10 mg/kg/day of a mixture of 40% AA I and 60% AA II for 35 days [7]. Nowadays, many authors prefer to use the more accurate term 'aristolochic acid nephropathy' (AAN) to the term CHN.

As a result of the Belgian CHN cases it became better known why certain Chinese THPs are contaminated with AAs and what the effects of exposure to AAs can amount to. Confusion over the Chinese common name 'Fang Ji', which can refer both to the roots of *S. tetrandra* and *A. fangchi,* could have caused the contamination with AAs [19, 20]. In the trade of Chinese herbs, the substitution of one plant species for another is established practice [23]. Besides the THPs known to contain *Aristolochia* species, some THPs derived of certain plant species can be identified that can be replaced by *Aristolochia* species in practice.

Several international Food and Medicine Authorities have published lists of THPs suspected to contain AA. The Dutch Food and Consumer Product Safety Authority (VWA) has implemented these lists in its sampling strategies of Chinese THPs and analyzed AAs in market samples. Reports providing quantitative data on AA levels of commercial THPs possibly containing AAs are scarce. This data could however be useful to validate the existing lists of suspected THPs and could help authorities to pinpoint their efforts to protect the consumer against exposure to AAs. In the present paper our results in this field over the past 4 years are presented and compared with recent scientific and regulatory data.

Materials and methods

Sampling

VWA inspectors sampled 190 Chinese THPs on the Dutch market in the period from November 2002 to June 2006. Samples were selected by using a list of single herb THPs and multi-ingredient THPs probably and possibly containing AAs, which was based on a list compiled by the FDA in 2001 [24]. The FDA list was supplemented and regularly updated in house with data on the nomenclature of AA containing herbal material from books on TCM, commercial TCM databases on the Internet, warnings of other inspection agencies and literature [1, 15, 23-28, 38, 40, 42].

Table 1 Examples of single herb THPs possibly containing AAs

Pin Yin name	part used	botanical name	Latin pharmaceutical name incl. synonyms
THPs with Aristolochia species			
Guang Fang Ji	root	*A. fangchi*	Radix Aristolochia fangchi/ Aristolochiae Fangchi
Ma Dou Ling	fruit	*A. contorta* and *A. debilis*	Fructus Aristolochiae
Tian Xian Teng	herb	*A. contorta* and *A. debilis*	Herba Aristolochiae/ Caulis Aristolochiae
Guan Mu Tong	stem	*A. manshuriensis*	Caulis Aristolochia manshuriensis/ Aristolochiae Manshuriensis
Qing Mu Xiang	root	*A. debilis*	Radix Aristolochiae
Xun Gu Feng, Bai Mao Teng	herb	*A. mollissima*	Herba Aristolochiae Mollissimae
THPs possibly contaminated with Aristolochia species			
Han Fang Ji	root	*Stephania tetrandra*	Radix Stephania tetrandra
Mu Fang Ji	root	*Cocculus trilobus* and *C. orbiculatus*	Radix Cocculi Trilobi/ Radix Cocculus trilobus/ Radix Cocculi
Chuan Mu Tong	stem	*Clematis armandii*	Caulis Clematis armandii/ Clematidis armandii
Chuan Mu Tong	stem	*Clematis montana*	Caulis Clematidis/ Clematis montana/ Clematis armandii
Bai Mu Tong	stem	*Akebia quinata*	Caulis Akebia quinata
Bai Mu Tong	stem	*Akebia trifoliata*	Caulis Akebia trifoliata
Bai Mao Teng, Bai Ying	herb	*Solanum lyratum*	Herba Solani Lyrati

References: [1, 23, 24, 27, 42]

Table 1 presents an extract of our sampling list defining the most pertinent *Aristolochia* species in use in TCM and several plant species with which they could be exchanged. The Chinese common names in the table refer to plants and the parts used and are given in Pin Yin, which is a phonetic representation of Chinese characters. The corresponding Latin pharmaceutical name also presented, is a combination of the part of the plant and often its binomial botanical name. Both types of nomenclature are seen in the market. Table 2 presents

examples of formulas, which can be potentially contaminated with AAs through the presence of Mu Tong, Fang Ji or Xi Xin. For sampling, products were selected by formula name or by screening the ingredient list for suspected herbs. Along with THPs known or suspected to contain AAs, THPs were sampled at random as well. THP names were copied as labeled and are presented in this paper without alterations. Generally no authentication of the herb was performed.

Table 2 Examples of multi ingredient THPs possibly containing AAs

Formula name in Pin Yin, in alphabetical order		
Formulas with Mu Tong or Fang Ji		
Anyang Jingzhi Gao	Fang Ji Huang Qi Tang	Quell Fire
Ba Zheng Wan	Fenqing Wulin Wan	Shang Zhong Xia Tong Yong Tong Feng Fang
Chi Kuan Yen Wan	Fu Ke Fen Qing Wan	Shi Xiang Fan Shen Wan
Chu Shi Wei Ling Tang	Gan Lu Xiao Du Dan	Shu Feng Huo Xue Tang
Chun Yang Zheng Ji Wan	Guan Xin Su He Wan	Shu Jing Huo Xue Tang
Da Huang Qing Wei Wan	Guo Qi Yin	Tienchi Hugu Wan
Da Qiang Huo Tang	Ji Jiao Li Huang Wan	Xiao'er Jindan tablets
Dang Gui Si Ni Tang	Ji Sheng Ju He Wan	Xiao Feng San
Dang Gui Si Ni Wan	Jia Wei Wu Lin San	Xiao Huo Luo Dan
Dao Chi San	Ju He Wan	Xiao Xu Ming Tang
Dao Chi Wan	Kat Kit Wan	Xin Yi Wan
Dieda Wan	Kuanhsin Suhowan	Xuan Bi Tang
Er Jia Jian Zheng Qi San	Long Dan Xie Gan Tang	Zhisou Huatan Wan
Ershiwuwei Songshi Wan	Long Dan Xie Gan Wan	Zhu Ling Tang
Fang Ji Fu Ling Tang	Mu Fang Ji Tang	
Formulas with Xi Xin		
Chuan Xiong Cha Tiao San	Du Huo Ji Sheng Tang	San Bi Tang
Chuan Xiong Cha Tiao Wan	Jiu Wei Qiang Hou Tang	She Gan Ma Huang Tang
Da Huang Fu Zi Tang	Ling Gan Wu Wei Jiang Xin Tang	Tong Guan San
Da Qin Jiao Tang	Ma Huang Fu Zi Xi Xin Tang	Wu Mei Wan
Dang Gui Si Ni Tang	Qu Feng Zhi Bao Dan	Xiao Qing Long Tang

References: [15, 24, 25, 27, 38, 40, 42]

THPs were collected in TCM stores, oriental food stores, wholesale dealers, importers or TCM practitioners throughout the Netherlands. Sampling inspections were held at least each year. Locations were selected from the VWA-inspection database. During inspections of these locations new suppliers and stores were also identified and visited. THPs were sampled in pre-packaged form in capsules or tablets or in many cases in the form of coarse herbal material from glass containers sometimes with limited or no labeling. Samples were taken on the basis of quantities supplied to consumers, which is one unit (e.g. bottle, package or container) or in

case of coarse herbal material in amounts higher than 10 gram. Mixtures of the coarse materials are assembled in TCM shops according to a formula prescribed by an in house TCM practitioner. This THP mixture is then prepared at home as a decoction for which the herbs are boiled in water or other liquids. The strained liquid is then consumed [1].

Experimental

The method used to quantify AA I and AA II was based on the method described by Flurer *et al*. [29]. The entire sample was homogenized or in case of capsules the contents of all the capsules was taken and homogenized, and from this a laboratory sample was taken for further analysis. After homogenization, 25 ml of extraction solution was added to 1 gram of sample. The extraction solution existed of 80% methanol, 18% water and 2% formic acid. The samples were shaken for 90 minutes in a shaking machine (Gerhardt LS-20, position 9). Then they were allowed to precipitate for about 1 hour. An aliquot of 1 ml was then centrifuged at a minimum of 10000 g. The supernatant was transferred to a vial and hermetically sealed for LC-MS analysis. No concentration step was needed.

Table 3 LC conditions for the determination of AA I and AA II

Parameters	Conditions		
Analytical column:	Alltima C-18 column (150 x 3.2 mm i.d. – 5µm particle size)		
Precolumn:	Alltima C-18 (7.5 x 3.0 mm i.d. – 5µm particle size)		
Column Temperature:	30°C		
Injection Volume:	20 µl (Full loop injection)		
Flow rate:	0.30 ml/min		
Mobile Phase:	Eluent A: 10 mmol ammonium formate in 1% formic acid		
	Eluent B: Methanol		
LC Gradient	Time (min)	Eluent A (%)	Eluent B (%)
	0.00	50	50
	10.00	20	80
	21.00	20	80
	22.00	0	100
	25.00	0	100
	26.00	50	50
	34.00	50	50

An Ion Trap LC-MS system of Thermofinnigan (LCQ Advantage) equipped with a quaternary pump, an autosampler with a column oven, a PDA-detector and an integration system together with LC-MS software was used for analysis. The separation was performed on an Alltima C-18 column (150 x 3.2 mm i.d. – 5µm particle size) with an Alltima C-18 precolumn (7.5 x 3.0 mm i.d. – 5µm particle size) using gradient elution. LC conditions

are listed in Table 3. The MS detection was performed by electrospray ionization (ESI) using the positive mode. The MS conditions are listed in Table 4. Before injection of samples, the system was equilibrated using 50% eluent A en 50% eluent B. Quantification of AA I and AA II was based on a standard mixture obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) containing 43% AA I and 54% AA II.

Table 4 MS conditions for the determination of AA I and AA II

Parameters	Conditions
Capillary temp:	250°C
Sheath gas:	45%
Aux/ sweep:	10
Source voltage:	5.00 KV
Source current:	80.00 µA
Capillary voltage:	9.00 V
Tube Lens offset:	15.00 V
Scan:	100.00 – 400.00 m/z
Parent ion AA I:	359.0 -> first daughter ion 298.0; second daughter ion 341.7 m/z
Parent ion AA II:	329.1 -> first daughter ion 267.9; second daughter ion 294.0 m/z

For every series of samples several quality checks were performed, including a check of validity of calibration, a check on the ratio of the first to the second daughter ion and the analysis of quality control samples (QC: 2 standardized control materials (SCM) containing both AA I or AA II). For this QC material the mean and a 95% confidence interval were established. Each measurement of this QC material had to comply with this 95% confidence interval. AA was only quantified when all quality checks for a series of samples were in line with the desired performance characteristics.

Results and discussion

Characteristics of the analytical method

For the in-house validation of the method for quantifying AA I and AA II, the limits of detection and quantification (LOD and LOQ) were determined. The LOD was defined as the concentration, which is three times higher than the range of the chromatographic background of the second daughter ion. The limit of detection determined in this way was 1.0 mg/kg for AA I and 1.6 mg/kg for AA II. The LOQ was defined as twice the LOD and was 2.0 mg/kg for AA I and 3.2 mg/kg for AA II. Two calibration curves were used, which both were linear with correlation coefficients of at least 0.97. The linearity for the low level AA ranges was determined between 86 - 430 ng/ml for AA I and 108 - 540 ng/ml for AA II. The range of application for preparations containing high levels of AAs was determined at 430-6450 ng/ml for AA I and 540 - 8100 ng/ml for AA II. The calibration curves for the high concentration range were established by quadratic regression. Preparations containing even higher amounts of AAs were diluted with the extraction solution.

The validity of the calibration curve, retention times and LOD were checked with each series of samples. Quantification of both AA I and AA II was based on the first daughter ions. However, amounts were only quantified when the relative intensity of the second daughter ion in proportion to the first daughter ion was within specific limits. For this the peak surface of the second daughter ion was calculated as a percentage of the peak surface of the first daughter ion. For AA I the relative intensity of the second daughter ion (m/z 341.7) had to be 44.0% ± 11.0% of the first daughter ion (m/z 298.0). Similarly for AA II the relative intensity of the second daughter ion (m/z 294.0) had to be 53.5% ±10.7% of the first daughter ion (m/z 267.9).

For the recovery studies a blank THP sampled from the market (labeled as Mu Tong and *Clematis armandii* and analytically shown to be free of AA I and AA II) was spiked at levels between 2 and 11 mg/kg. As Mu Tong samples were expected to be frequently contaminated, this blanc Mu Tong sample without AAs was considered to be a relevant model for our research and the recovery studies were therefore performed on this sample. The recoveries determined from six replicate measurements under repeatability conditions were 87% and 82% respectively for AAI and AAII with relative standard deviations (RSD) of 3.7 and 3.5% respectively. The reproducibility was defined as 2.8 times the standard deviation obtained from results determined by different operators and at different times using standardized control materials (SCM).

For AA I a SCM, a Xi Xin THP sampled from the market containing 4.75 mg/kg AA I, was used to determine the reproducibility which amounted to 0.94 mg/kg. For AA II a SCM was prepared from a mixture of a Guan Mu Tong THP sampled from the market (an *Aristolochia* sp. with a high level of AA II) and the blanc Mu Tong sample without any AAs which was used for the recovery studies. This mixture contained 3.76 mg/kg AA II and the reproducibility was 0.47 mg/kg. Both SCMs were analyzed each series. These performance characteristics of the method are in agreement with results published by Trujillo *et al*. [30].

Confusion of herbs in TCM and sampling of AA containing THPs

Besides a sensitive analytical method, an effective sampling protocol needed to be developed for enforcement of the ban on AAs. Central to this protocol was information on which Chinese herbs can possibly be replaced by herbs that contain AAs. In TCM confusion of herbs occurs frequently and can result from similarities in appearance, mistakes in (ancient) textbooks, counterfeits and in many cases ambiguous nomenclature [31]. Contamination of THPs with AAs can often be traced back to confusion over nomenclature. Common or vernacular names of plants are, as opposed to binomial botanical names, not very reliable for unambiguous identification of the particular species as, for instance, the interpretation of common names can even differ between geographical regions.

In TCM, several plant species share a Chinese common name with an *Aristolochia* sp. and this common name could be seen as a group name for the species concerned. When a prefix is added to the group name, the common name refers to only one or two plant species of the group; in many cases however only the group name is used. The prefix can point at a region where the plant is grown, for example the prefix Chuan refers to the Sichuan province [1]. The common name Fang Ji refers to at least 4 plant species but in combination with the prefix 'Guang' it is exclusively used for the root of *Aristolochia fangchi*. There is also the possibility that a herb has

more than one common name which can lead to confusion as well. For instance, it was recently reported that *Aristolochia mollissima* is not only called Xun Gu Feng but Bai Mao Teng as well. This last common name is also used for *Solanum lyratum*, which confusingly has an alternate name as well, namely Bai Ying [32]. Substitution of *S. lyratum* by *A. mollissima* can occur when only the common name Bai Mao Teng is used when the THP is prescribed, self medicated, traded etc. Such a case has been reported in Hong Kong recently where a 60-year-old man was diagnosed with renal failure and urethral cancer after he had erroneously been using Herba Aristolochia mollissimae instead of the desired *Solanum* species [31]. The authors indicated that confusion keeps recurring between the names Xun Gu Feng, Bai Mao Teng and Bai Ying [31]. Although *A. mollissima* is entered in the FDA list, the plant's common name is not mentioned nor is *S. lyratum* [24]. Information of this nature might prove valuable when sampling THPs for AA analysis.

Tables 1 and 2 present respectively an extract of our sampling list defining the most pertinent single herb THPs derived from *Aristolochia* spp. in use in TCM, several plant species which could be replaced by *Aristolochia* spp. and a list of possibly contaminated formulas. This list was proven useful for sampling 190 Chinese THPs on the Dutch market in the period from November 2002 to June 2006.

Analytical results

The Chinese common names and Latin pharmaceutical names of all 190 THPs were examined for indications that AA containing herbs might be present. We identified 68 THPs as products that could possibly contain AAs and this subgroup contained all 25 positive samples. The analytical results of the 68 potentially AA containing THPs are presented in Table 5 grouped by the Chinese common name. AA I was found in all 25 samples positive for AA, the AA I level of 4 THPs was below the LOQ and AA I contents of the remaining samples ranged between 2 mg/kg and 1676 mg/kg. Together with AA I, AA II was detected in 13 samples with the AA II content of 1 sample below the LOQ and that of the other samples between 4 mg/kg and 444 mg/kg. When THPs contained more than the relevant LOQ action was taken to remove the products from the market.

In 3 of 5 samples of single herb THPs (Guan Mu Tong $^{(2x)}$, Qing Mu Xian, Guang Fang Ji and Tian Xian Teng) labeled with names exclusively referring to *Aristolochia* species, significant amounts of AA I were found ranging between 74 and >1000 mg/kg implying that these samples contained indeed *Aristolochia* spp. The remaining 2 of these *Aristolochia* samples surprisingly contained no detectable levels of AAs. The identity of the samples was generally not authenticated however. In 11 of 12 samples of THPs with herbs from the genus *Asarum*, which also belongs to the *Aristolochiaceae* and in which AAs can be expected, low levels of AAs were detected. The remaining 11 positive samples were THPs that contained herbs, which can be substituted by *Aristolochia* spp. and belonged to the Fang Ji and Mu Tong groups of Table 5. Of these, 4 products were incorrectly labeled with names that identified the herbs as AA free counterparts of *Aristolochia* species. This shows that the problem in TCM of substitution of innocuous herbs with *Aristolochia* spp. is not yet resolved.

Table 5 LC-MS detection of Aristolochic acids (AAs) in Chinese THPs potentially containing AAs sampled on the Dutch market

Chinese common name		Latin Pharmaceutical name,	N	N	AA (mg/kg) in positive samples			
Group	Specific names, when labeled	when labeled	without AA	with AA	AA I	Mean AA I	AA II	Mean AA II
Mu Tong	Guang Mu Tong	---		2	919; >1000		82; > 100	
	Guang Mu tong	Caulis Clematidis Armandii	1		n.d.		n.d.	
	Mu Tong	---		1	1453		303	
	Mu Tong [2x]/--- [3x]	Caulis Clematidis Armandii - Akebiae/ Caulis Clemat(id)is Armandii [4x]	5		n.d.		n.d.	
	Mu Tong	Caulis Akebiae		1	1281		394	
	Chung Mu Tong/ Chuan Mu Tong[2x]/ Chuang Mu Tong	Caulis Akebiae[4x]	1	3	19; 1113; 1676	936	4; 190; 444	212
Mu tong (in formula)	Lon Dan Xie Gan (Pian)/ Long Dan Xie Gan Tang	---/ Gentiana form	4	3	41; 49; 59	50	11; 9;14	11
	---	Quell Fire	1		n.d.		n.d.	
	Xao Feng San	Tangkuei & Arctium Formula	1		n.d.		n.d.	
	Dao Chi San	Rehm. Clematis Armandi Form.	1		n.d.		n.d.	
	Xiao Feng San	Tangkuei & Arctium Formula	1		n.d.		n.d.	
Mu Xiang	Qing Mu Xiang	Radix Aristolochiae, “Duitse pijp”	1		n.d.		n.d.	
	Mu Xian	---	1		n.d.		n.d.	
	Mu Xiang	Vladimiria	1		n.d.		n.d.	
	Guang Mu Xiang	---	1		n.d.		n.d.	
Tian Xian Teng	Tian Xian Teng	Caulis Aristolochiae		1	74		33	

Table 5 (continued)

Chinese common name		Latin Pharmaceutical name	N	N	AA (mg/kg) in positive samples			
Group	Specific names, when labeled	when labeled	without AA	with AA	AA I	Mean AA I	AA II	Mean AA II
Fang Ji	Niu Ru Shi	Cocculi Sarm. Rad.		1	< LOQ		nd	
	Guang Fang Ji	---	1		nd		nd	
	Fang Ji	---	1		nd		nd	
	Fanji	Sclerotium Poriae Cocos		1	12		< LOQ	
	Fang Ji/ ---	Radix Stefaniae Tetrandiae (2x)	2		nd		nd	
Fang Ji (in formula)	Fang Ji Qi Tang	Wutian & Astrag. Comb	1		nd		nd	
	Mu Fang Ji Tang	Gypsum, Cinn & Ginseng Comb		1	524		21	
Xi Xin	Xi Xin (Bei) (1x)/ Xi Xin	Herba Asari (North) (2x)/ Herba Asari/ Herba Asari Cum Radice	1	10	< LOQ – 31	9	nd	
Xi Xin (in formula)	Dang Gui Si Ni Tang	Tangkuei & Jujube		1	< LOQ		nd	
Wei Ling Xian	Wei Ling Xian	Clematis/ Radix Clematidis/ Radix Clematidis Chinensis	10		nd		nd	
	---/ ---	Clematidis/ Radix Clematidis	5		nd		nd	
Wei Ling Xian (in formula)	Shu Jing Huo Xue Tang	Clematis & Peony Comb	1		nd		nd	
Ba Yue Zha	Ba Yue Zha	Fructus Akebiae	2		nd		nd	

The combinations of Chinese common names and Latin pharmaceutical names were copied from the label or provided by the vendor. No translations of Chinese names into Latin pharmaceutical names were added by the authors. Chinese common names with small differences, which were deemed synonyms, were grouped.

nd: not detected

(---) indicates that a name was not present on the label or that the vendor could not specify the name

The problem of substitution of Chinese herbs with toxic counterparts is not limited to *Aristolochia* spp. In 2001 the Dutch Health authorities including the VWA were faced with more than 60 poisonings with symptoms including epileptic seizures due to consumption of a herbal tea where the spice Chinese star anise (*Illicium verum*) was replaced by an unidentified *Illicium* sp. imported from China and which was shown to contain the neurotoxin anisatin [33].

We found no AAs in THPs which to our knowledge were not likely to be contaminated with AAs and could therefore be considered as randomly sampled. These THPs are presented in Table 6.

Table 6 Randomly sampled Chinese THPs negative for Aristolochic acids (AAs) as determined with LC-MS

Chinese common name, as labeled	Latin Pharmaceutical name, as labeled
Ba Zhen Wan	---
Ba Zheng Ke Li	Octo Form granules
Bai Hua She She Cao	Herba Hedyoti Diffusae
Bai Qui Feng	---
Bao He Wan	---
Bi Xie	Rhizoma Dioscorea Hypoglaucae
Bian Dou	Semen Lablab Album
Bing Lang	---
Cang Zhu	Rhizoma Atracttylodi
Che Qian Cao (2x)	Plantago Asiatica/ Herba Plantagaginis
Chuan Wu/ Chuang Mu/ ---	R. A. Carmichaeli (2x)/ Aconit Carmichaeli Preparata
Da Huang (2x)	---
Dang Gui	Chinese Angelica
Dao Chi Pian	Scarlet Form
Ding Chuan Wan	---
Du Zhong Bu Yao Granules	---
Fan Xie Ye	Folium Sennae
Fang Feng	Radix Sapashnikovae S
Fu Ling (3x)/ --- (2x)	Sclerotium Poriae Cocos (5x)
Fu Ping	---
Gui Fu Di Huang Wan	---
Guo Teng	Rumulus Unicare Cum Uncis
He Shou Wu (2x)/ ---	Radix Polygoni Multiflori (3x)
Hong Hua	Flos Carthami Tinctorii
Huai Hua	Flos Sophorae
Huang Jing	Rhizoma Polygonati, Polygonum officinale
Huang Lian Tang Granules	---
Huo Xiang Zheng Qi Wan	---
Je Yiao Teng	Caulis Polygoni Multiflori

Table 6 (continued)

Chinese common name, as labeled	Latin Pharmaceutical name, as labeled
Jian Pi Wan	---
Jinkuishenqiwan	---
Ku Shen Pian	---
Kuan Dong Hua (3x)/ --- (2x) / Dong Hua	Flos Tussilagi Farfarae (6x)
Lei Gong Teng	Herba Polygoni Perfoliali
Li Zhong Ke Li	Midrif Form granules
Ma Chi Xian	Herba Portulacae
Mai Wei Di Huang Wan	---
Mi Niao Ning Ke Li	---
Mi niao ning ke li	---
Qing Fei Ping Chuan Tang Granules	---
Qing Qi Hua Tan Pian	---
Quang Huo Rhizoma	Radix Notop Tergii
Ren Shen Ye	Folium Ginseng
Sang Ju Yin Ke Li	Chrysanth Form
Sang Zhi	---
Shenzhi Jiaonang	---
Shu Gan Wan	---
Shugawan	---
Su Mu	Lignum Sappan
Te Xiao Yao Tong Ling	---
Tiang Huang	---
Wu ji bai fe	---
Wu ji bai feng wan	---
Wu Yao	R. L. Strychnifoliae
Xi Zhi Ren	Black Cardamom
Xia Sang Ju Chong Ji	---
Xiang Yuan	Flos C. Mediae
Xiao Feng Ke Li	Lay Wind Form
Xiao Ji	---
Xiao Yao San	Tangkuei & Bupleurum Formula
Ya Dan Zi	Fructus Brucae Jav
Yan Fu Mu	---
Yang Xue Sheng Fa Jiao Nang	---
Ye Jiae Tang	---
Yin Chen	Herba Artemisiae Scopariae (2x)
Yu Mi Xu	Stigma Maydis
Yu Zhu (2x)/---	Rhizoma Polygonati/ Polygonati Odorati (2x)

Table 6 (continued)

Chinese common name, as labeled	Latin Pharmaceutical name, as labeled
Ze Lan/ ---	Herba Lycopi (2x)
Zi Cao	Radix Arnebiae S. Lithosspermi (2x)
Zi Cao (2x)	Radix Arnebiae (2x)
Zi Hua Di Ding	Herba Violae
Zuo Gui Wan	---
---	Aconite Ginseng & Ginger Combination
---	Artemisia Scoparia
---	Astragalus extract tablets
---	Beautifying and slimming tea
---	Bupleurum & Dragon Bone Combination
---	Flos Chrysanthemi (3x)
---	Herba Artemisiae Annuae
---	Herba Eupatorii Fortunei (2x)
---	Herba Lobeliae Chinensis Cum Radice (2x)
---	Plantaginia Semen extract
---	Radix Phytolaccae
---	Radix Pulsatilae Chinenses
---	Radix Rubiae
---	Rehmannia Eight Formulas
---	Rhizoma Dryopteria Crassihizomae (2x)
---	Taraxaci Herbs

Though AAs were absent, some of these products could pose a health risk to the user because they may contain other natural toxins. For example, we sampled 2 THPs labeled as Chuan Wu and Radix Aconiti Carmichaeli, which is the root of *Aconitum carmichaeli*. This plant and the related *A. kusnezoffii* (Cao Wu) are used in TCM for the treatment of musculoskeletal disorders and contain the potent neurotoxin and cardiotoxin aconitine. In Hong Kong cases of herb-induced aconitine poisonings are treated almost every year [34]. Also several herbs were sampled known to contain pyrrolizidine alkaloids (PAs), which are mutagenic and carcinogenic hepatotoxicants (for a review see [12]). The PA containing herbs are Zi Cao (*Arnebia euchroma* and *Lithospermum erythrorhizon*), Kuang Dong Hua (*Tussilago farfara*) and Pei Lan (*Eupatorium fortunei*) [35]. Altogether, based on our results it can be concluded that especially single herb THPs under the Chinese common names of Mu Tong, Fang Ji, Tian Xian Teng and Xi Xin could be contaminated with AAs. Besides single herb THPs, certain multi-ingredient THPs can be at risk of adulteration as well (Table 5). In the following paragraphs we will expand on the possible reasons of contamination of these particular THPs with AAs.

Mu Tong

We found AAs in 7 out of 14 single herb THPs with the pharmaceutical names Caulis Akebia or Caulis Clematis armandii or Mu Tong with or without the prefixes Guan or Chuan. The common Chinese name Guan Mu Tong exclusively refers to the stem of *Aristolochia manshuriensis* [23] and 2 samples exclusively labeled as "Guang Mu Tong" [*sic*] contained an AA I level of 919 mg/kg and 1000 mg/kg or higher and the AA II contents were 82 mg/kg and > 100 mg/kg respectively. Hashimoto *et al*. [3] report AA I contents of *A. manshuriensis* (Kan-mokutsu in Japanese) ranging between 0.169 - 0.882 %, which is more than 1.5 - 8.8 times higher than the levels we found. Trujillo *et al*. [30] reported an AA content of 2830 mg/kg in a sample of *A*. *manshuriensis* stem. In our study another sample verbally indicated to be "Guang Mu Tong" [*sic*], but labeled as "Caulis Clematidis Armandii", contained no AAs. The stem of *Clematis armandii* is called Chuan Mu Tong, however. We therefore conclude that the prefix Guang was mistaken for Chuan, which would explain the absence of AAs. A further 5 samples labeled as Caulis Clematidis Armandii with or without Mu Tong, did as expected not contain AAs. Of the positive samples, 3 were labeled as a Chuan Mu Tong and Caulis Akebia (stem of *A*. *quinata* or *A*. *trifoliata*), which to our knowledge is not a common combination. The common Chinese name of Caulis Akebia is Bai Mu Tong and not Chuan Mu Tong, which refers to the stem of Clematis armandii, and *C*. *montana* [1, 23]. Furthermore, 2 THPs labeled as Mu Tong, one of which also labeled as Caulis Akebiae, contained high levels of both AA I and AA II. These findings strongly underline that the nomenclature of THPs cannot to be relied upon in some cases. Also the fact that AAs were found in half of the Mu Tong samples indicates that this group of THPs needs constant monitoring. Bensky & Gamble note that in premodern China *Akebia* was used as Mu Tong but that at present *Aristolochia manshuriensis* is used most often [1].

The formula Long Dan Xie Gan Wan is included in the FDA list of potential AA containing herbs and formulas [24]. The suffix "Wan" in the formula name refers to pill in Pin Yin [1]. The formula Long Dan Xie Gan Tang is reported to contain 10 ingredients among which Caulis Mu Tong [25] for which either *Aristolochia manshuriensis* or the known *Akebia* spp. and *Clematis* spp. can be used [1]. In our study, we sampled Long Dan Xie Gan and related products with the suffixes "Pian" (tablet) and "Tang" (decoction) [1, 25] and found 41-59 mg/kg AA I in 3 of 8 related samples. Health authorities such as Health Canada and the MHRA in the UK issued warnings against the use of this product in 2002 and 2003 [15, 16]. Synonyms are "Quell fire" and "Lung Tan Xie Gan pills" [36]. Quell fire tablets were taken of the market in 2000 and reformulated as requested by the FDA after the detection of AAs [37]. We found no AAs in one sample of Quell fire, which had a different lot number and expiry date than the earlier recalled lots [37]. A case of end stage renal failure and recurrent carcinomas in the bladder due to the 5-year use of Longdan Xieganwan manufactured in China, was reported in the UK [38]. The formulas Dao Chi San and Xiao Feng San were sampled each once and were found to be negative for AAs in our study. Both formulas contain Caulis Mu Tong but some of the classical sources describe Xiao Feng San without this herb however [25]. Xiao Feng San has been found to contain AAs in Australia [17]. The FDA included the formula Dao Chi Wan in the listing of THPs suspected to contain AAs [24]. This formula has probably the same composition as Dao Chi San except that the latter is a powder (San) instead of a pill (Wan) [25]. It is prudent to

include the formulas Long Dan Xie Gan, Dao Chi San and Xiao Feng San in a sampling protocol because of a possible inclusion of *A. manshuriensis*.

Mu Xiang and Tian Xian Teng

No AAs were detected in 4 related Mu Xiang samples. This common name can refer to the roots of *Aristolochia debilis, Aucklandii lappa, Saussurea lappa, Inula helenium, I. racemosa* and *Vladimiria souliei* [23]. Qing Mu Xian exclusively refers to the root of *Aristolochia debilis* (see Table 1) but a sample of this THP contained contrary to expectations no AAs. Although our samples of Mu Xian, Guang Mu Xian (the root of *Saussurea lappa*) and Vladimiria (Chuan Mu Xian is the root of *V. souliei* [1]) could potentially be substituted by *A. debilis* we did not detect AAs. This is in agreement with information of the European Agency for the Evaluation of Medicinal products (EMEA) [23]. More research is needed however to evaluate the likelihood of this particular substitution. Another single herb THP originating from an *Aristolochia* sp. is Tian Xian Teng, which according to the Chinese Pharmacopoeia is Herba Aristolochiae, derived of *A. contorta* and *A. debilis*. A sample of this THP was found to contain 74 mg/kg of AA I and 33 mg/kg of AA II. Although Tian Xian Teng was sampled once and might not be very common on the market it should be included in sampling protocols.

Fang Ji

In TCM the common name Fang Ji generally refers to several different herbs, namely *Cocculus trilobus, C. orbiculatus*, *Stephania tetrandra* and *Aristolochia fangchi*. Guang Fang Ji refers exclusively to *Aristolochia fangchi* and is the only of these species to contain AAs [1, 23]. We sampled 1 THP verbally indicated as Guang Fang Ji, which contained no AA however. A low concentration of AA was detected in a sample labeled as Fanji and Sclerotium Poriae Cocos. In a THP called Niu Ru Shiu, unknown to us, but also labeled as Cocculi Sarm. Rad. traces of AA I were detected. Obviously Rad. stands for Radix and in the FDA list the herbs *Cocculus sarmentosus* and *C. trilobus* are included as synonyms of *Cocculus orbiculatus* [24]. The root of *C. orbiculatus* as well as *C. trilobus* is called Mu Fang Ji in several sources [1, 39] and might therefore be substituted with Guang Fang Ji (*Aristolochia fangchi)* (Table 1) [24]. We found a relatively high AA I concentration of 523 mg/kg and 21 mg/kg of AA II in the multi ingredient THP Mu Fang Ji Tang, which contains Radix Cocculi Trilobi (Mu Fang Ji) or Radix *Stephania tetrandra* (Han Fang Ji) according to some sources [40, 41] or Radix *Aristolochiae fangchi* to another source [42]. We found no AAs in 3 single herb THP samples labeled as Radix *Stephania tetrandra* and/or Fang Ji. This does not indicate however that Fang Ji requires less attention. An AA contaminated THP labeled as *Stephania tetrandra* was the cause of the Belgian AAN incident [20-22]. In a Swiss survey of AA I in commercial samples of slimming regimens consisting of Chinese plant mixtures, 4 out of 42 were tested positive. AA I was found in a sample Han Fang Ji declared to be Stephania tetrandra radix and traces were found in Han Fang Ji derived of *Sinomenium acutum* [28]. Both species are listed in the FDA list [24]. Another multi-ingredient THP analyzed by the Swiss researchers called Fang Ji Huang Qi Tang contained traces of AA I but a second sample did not [28]. We could not detect AAs either in Fang Ji Qi Tang, which could be a related formula. More attention

should be focused on multi ingredient THPs and the lists of these THPs potentially containing herbs with AAs should be expanded.

Xi Xin

We found low levels of AA I ranging between the LOQ and 31 mg/kg in 10 out of 11 samples labeled as Xi Xin, Xi Xin (Bei) and Herba Asarum or Herba Asarum (North), Herba Asarum cum Radice samples. No AA II was found. Xi Xin refers to *Asarum sieboldii*; the genus *Asarum* belongs to the *Aristolochiaceae* and could be expected to contain AAs. Hashimoto *et al*. [3] analyzed the Chinese *Asarum* spp. *A*. *heterotropoides*, *A*. *sieboldii*, *A*. *splendens* and *A*. *himalaicum* and *A*. *forbesii* and found only traces of AA I in *A. splendens* and *A*. *himalaicum*. Schaneberg *et al*. [4] found up to 370 mg/kg AA I but no AA II in the North American *Asarum* species *A*. *canadense*. Although the AA levels found in this study are low, batches of Xi Xin should be routinely screened for AAs before they are brought on the market.

Wei Ling Xian and Ba Yue Zha: likelihood of substitution by Aristolochia spp.

In the FDA list Wei Ling Xian or the root of *Clematis chinensis* is included as a THP, which may be adulterated with AA [24]. The Latin pharmaceutical name is Radix Clematidis and also refers to the roots of *C. hexapetala* and *C. uncinata*. Substitution of these herbs by the stem of *Clematis armandii* or *C. montana* (Chuan Mu Tong which can be replaced by the stem of *Aristolochia manshuriensis,* Guan Mu Tong) (seems not likely because the root of these last Clematis spp. is not reported to be in use for Wei Ling Xian [1, 43]. Another hypothetical option is confusion between the Wei Ling Xian and Qing Mu Xian (the root of *Aristolochia debilis*) but we found no reports of this substitution. We analyzed 10 samples of Wei Ling Xian/ Radix Clematidis (Chinensis), 5 samples of Radix Clematidis and a formula containing this herb [25] but no AAs were found. Our results might indicate that Radix Clematidis or Wei Ling Xian is not likely to be replaced by *Aristolochia* spp. Nevertheless the FDA reported in 2001 the contamination of a *Clematis chinensis* extract with AAs [44], more research into this substitution is therefore warranted.

Another herb where substitution by *Aristolochia* spp. seems unlikely is Fructus Akebiae or Ba Yue Zha. According to Bensky & Gamble [1] both names refer to the fruit of *Akebia quinata* and *A. trifoliata*. The fruit of these plants has also been reported to be referred to as Yu Zhi Zhi in the Pharmacopoeia of the People's Republic of China and is entered as such in the FDA list [24, 45]. We found no AAs in 2 samples labeled as Fructus Akebiae and Ba Yue Zha. As the common Chinese name of Fructus Akebiae bears no resemblance to a common name of any *Aristolochia* sp. confusion would not seem likely. This has to be supported by additional results however, before this specific THP may be considered for removal from the list of suspected materials. It might be possible that the fruit of *Aristolochia debilis* or *A*. *contorta* (Ma Dou Ling) and Fructus Akebiae are used interchangeably but we are not aware of reports of such a substitution.

Exposure data

The batches of *Stephania tetrandra* powder which were replaced by *Aristolochia* spp. in the Belgian AAN incident were reported to contain AA levels up to 1.56 g/kg with a mean of 0.65 g/kg [20]. Interestingly only in 2 of the 12 investigated batches tetrandrine, the characteristic alkaloid of *S. tetrandra*, was found and in 1 of these in combination with AAs. This would indicate that the rest of the batches consisted of 100% *Aristolochia* spp. replacing *S. tetrandra* [20]. It was estimated that the cumulative consumption of more than 200 g of these powders raised the risk of urothelial carcinoma [21]. This corresponds to a cumulative chronic intake of 130 mg of AAs when using the mean AA content reported by Vanhaelen *et al.* [20]. We sampled a THP labeled as Chuan Mu Tong and C. Akebia on the Dutch market with an AA content of 2.1 g/kg consisting of a natural mixture of 1.7 g/kg of AA I and 0.44 g/kg of AA II (Table 5). In a worst-case scenario 62 g of this THP with the highest AA content would supply more than 130 mg of AAs that, considering the Belgian data, could significantly raise the risk for cancer. According to Bensky & Gamble the recommended dosage of Mu Tong is 3-9 g, probably per day [1]. The authors warn against overdose with reference to a case of acute renal failure following a dose of 60 g. No limitation in the duration of use is given. When in our worst case scenario the preparation with the highest AA content would be used following the highest dosing regimen, exposure to more than 130 mg of AAs would be achieved in 7 days assuming that all AA is released from the matrix. In the Belgian cases of urothelial carcinoma, the mean exposure duration was 15 months and generally end-stage renal failure occurred 3 to 85 months after cessation of the herbal regimen [21]. In Belgium, the Fang Ji was consumed as a powder, which might have increased the exposure to AAs. In China however, Chinese THPs are mostly used as decoctions, which might reduce the toxicity of *Aristolochia* spp. However in Chinese literature 2 cases of acute renal failure after consuming a decoction made of 70 g and 175 g of Mu Tong, probably Guan Mu Tong and 4 deaths of renal failure after consuming decoctions of 50-120 g of Mu Tong were reported [46, and the references therein]. In the UK, 2 cases were reported of end stage renal failure following the use of Mu Tong containing AA I and AA II. Mu Tong was consumed by one patient as a tea for 6 years and the other patient used a preparation for 2 years in an undisclosed way [47].

Conclusion

Our finding that AAs were detected in 25 of 68 THPs sampled on the Dutch market, which could include AA containing herbs, indicates that several years after the ban the risk of inadvertent exposure to AAs remains significant for those who use these particular THPs. In 1999, the MCA, now the MHRA, found AAs in 40% of the samples with Fang Ji and Mu Tong on the UK market [23]. Although the number of samples is relatively small we found a similar percentage of samples containing AAs namely 37 % of the suspected samples, which indicates that the situation has not improved since. In the UK the use of Mu Tong, Fang Ji, Ma Dou Ling or Qing Mu Xiang is prohibited since 2001. Amongst these are also species such as *Stephania tetrandra, Clematis* spp. and *Akebia* spp., which do not belong to the *Aristolochiaceae* and do not contain AAs [15]. The Dutch Commodities Act Decree 'Herbal preparations' used to prohibit the sale of *Magnolia officinalis* and *Stephania tetrandra* as well.

After a re-evaluation of the literature it was concluded that these herbs in themselves pose little risk and subsequently the prohibition of these herbs was discontinued but it was recognized that a risk of substitution of *S. tetrandra* by *Aristolochia fangchi* remained [48].

Internationally, the problem of AA contaminated THPs still requires attention several years after measures by regulatory authorities in countries as Great Britain, New Zeeland, Canada and Australia and publicity generated by this [14-18]. The Belgian AAN tragedy clearly illustrates that contamination of THPs with AAs can have very serious consequences. Continued enforcement of the ban of AAs in the Netherlands will show if the problem of AA contamination of Chinese THPs is addressed more actively in the field of trade and if stricter regulatory measures are warranted. When identified, contaminated products will be removed from the Dutch market. The VWA will also in collaboration with customs direct its enforcement at the import of herbal material in order to prevent AA containing THPs from entering the market. More research into possible contamination of THPs will help to safeguard the quality of Chinese THPs. As contamination is unnecessary TCM practitioners, manufacturers, vendors and importers of Chinese THPs should structurally direct efforts to the avoidance of AAs in THPs known to have the potential of contamination with AAs. Certification of THPs might aid to prevent the import of AA-contaminated products but such a system needs close monitoring. It can be concluded that testing of the imported herbs for AA contamination is still essential.

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CHAPTER
5

Based on:

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Monitoring of mercury, arsenic, and lead
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and estimation of associated risks

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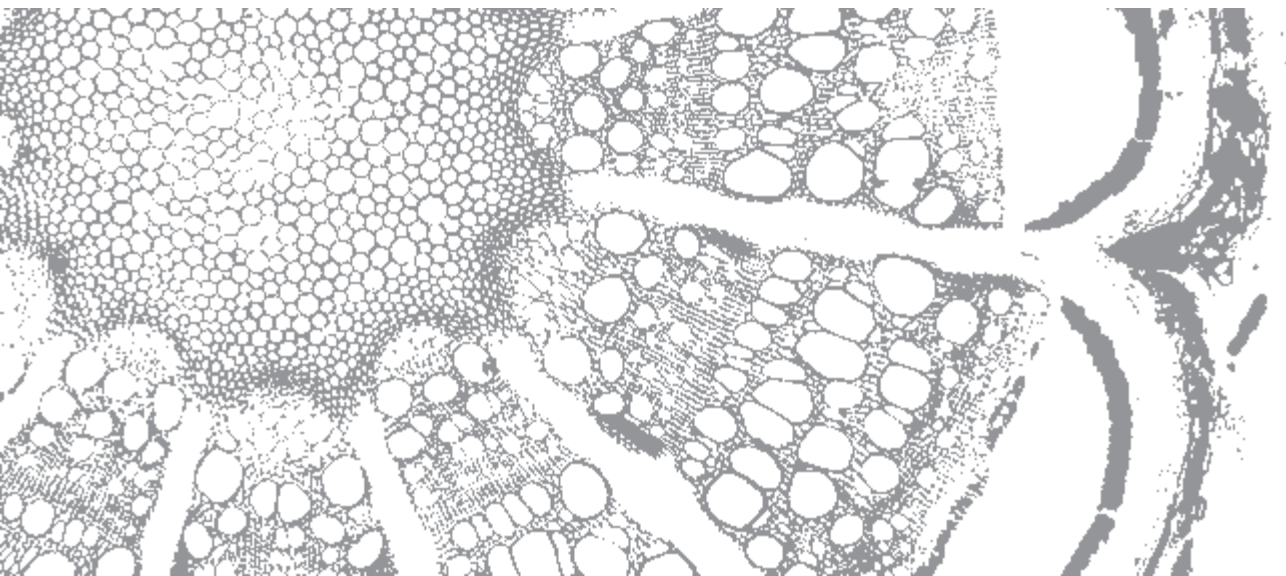
Monitoring of mercury, arsenic and lead in traditional Asian herbal preparations on the Dutch market and estimation of associated risks



Abstract

Traditional herbal preparations (THPs) used in Ayurveda, Traditional Chinese Medicine (TCM), Traditional Tibetan Medicine (TTM) and other Asian traditional medicine systems may contain significant amounts of mercury, arsenic or lead. Though deliberately incorporated in Asian THPs for therapeutic purposes, these constituents have caused intoxications worldwide. The aim of this study was therefore to determine mercury, arsenic and lead levels in Asian THPs on the Dutch market. 292 THPs used in Ayurveda, TCM and TTM were sampled between 2004 and 2007. Samples were mostly multi ingredient THPs containing herbs and minerals. The labeling of less than 20% of the THPs suggested the presence of mercury, arsenic or lead. These elements were shown by ICP-MS in 186 (64%) of 292 THPs. Estimated weekly mercury, arsenic and lead intake levels were calculated for each THP from the analytically determined concentrations and the recommended dose. 59 THPs (20%) were likely to result in intakes of these elements significantly exceeding safety limits. Of these 59 THPs, intake estimates for 50 THPs significantly exceeded the safety limit for mercury (range 1.4-1747 mg/week); intake estimates for 26 THPs significantly exceeded the safety limit for arsenic (range 0.53-427 mg/week) and intake estimates for 8 THPs were significantly above the safety limit for lead (range 2.6-192 mg/week). It is concluded that the mercury, arsenic and lead contents of THPs used in Ayurveda, TCM and TTM remain a cause for concern and require strict control.

Introduction

In Asian traditional medicine systems such as Traditional Chinese Medicine (TCM), Traditional Tibetan Medicine (TTM) and the Indian traditional medicine systems Ayurveda, Unani-Tibb and Siddha, traditional herbal preparations (THPs) play an important role [1-4]. In the Netherlands, Asian THPs can be brought on the market as food supplements, which do not require prior safety evaluation. Asian THPs have been reported to contain herbal toxic principles, undeclared synthetic drugs and significant amounts of mercury, arsenic and lead [5-14]. Use of Indian, Tibetan and Chinese THPs has resulted in lead poisonings worldwide and several mercury and arsenic intoxications as well [15-31]. In Ayurveda, Siddha, Unani-Tibb, TTM and TCM, ingredients high in the metals mercury and lead or the metalloid arsenic are added to THPs for therapeutic purposes [1, 2, 4, 16, 18]. In TCM tranquilizing and detoxifying effects are attributed to mercury [16] and nearly 10% of the formulas in the Pharmacopoeia of China incorporate the mercury containing mineral cinnabar [32, 33]. In 25% of the 634 formulas for Ayurvedic THPs in the official Ayurvedic Formulary of India, mercury, lead and arsenic compounds are listed as ingredients [34, 35]. An Ayurvedic discipline called 'Rasa shastra' is dedicated to the study of the therapeutic use of mercury and other metals or metalloids [36]. In most cases high levels of metals or metalloids in Ayurvedic THPs result from intentional incorporation of certain metallic preparations called 'bhasmas' in the products. The metals and metalloids in bhasmas are claimed to be detoxified by elaborate processing steps including many cycles of heating and subsequent cooling in herbal mixtures and animal products such as cow's urine or ghee [18, 34, 35, 37, 38]. Comparable preparations called kushtas and parpams are in use in Unani-Tibb and Siddha, respectively [1, 2].

Metals and metalloids can exist in different defined chemical species and the toxicity of these species can vary significantly [39]. Available reports consistently point to the presence of inorganic metal or metalloid compounds in Chinese, Tibetan and Indian THPs including Ayurvedic THPs. Naga bhasma, a widely used Ayurvedic lead preparation [34], contains lead sulfide (PbS); other lead preparations contain lead oxide (PbO) or lead sulfate ($PbSO_4$) [37, 38]. In TCM, lithargyrum or 'mi tuo seng' (PbO) is used [40]. Important Ayurvedic mercury preparations are 'parada' (purified mercury), 'hingula' (cinnabar, HgS), 'kajjali' (HgS) and 'makaradhvaja' (HgS) [38,41]. In TTM, processed mercury preparations (tsothel) are used as well, which mainly consist of mercuric sulfide (HgS) and smaller amounts of mercuric sulfite ($HgSO_3$) and mercuric sulfate ($HgSO_4$) [4]. In TCM, mercury is used as cinnabar (zu sha) and as calomel (qing fen, Hg_2Cl_2) [10, 16, 40, 42, 43]. In both Chinese and Ayurvedic THPs, realgar (As_4S_4), orpiment (As_2S_3) and arsenolite (As_2O_3) are used but in Ayurveda these minerals are processed before use [10, 40, 42-44]. In certain kushtas (used in Unani-Tibb) As_2O_3 was found, but kusthas can contain other inorganic arsenicals as well [2, 17, 45].

In 2004, the Dutch Food and Consumer Product Safety Authority (VWA) received reports of two cases of lead poisoning linked to the use of Ayurvedic THPs from, respectively, India and Nepal [46, 47]. These cases prompted the VWA to intensify investigations into metal and metalloid contents of Asian THPs. The objective of this investigation was to study the metal and metalloid contents of Asian THPs on the Dutch market and assess

the related risks by comparing the estimated metal and metalloid intake resulting from use at the proposed dose levels to established toxicological safety limits. The current report presents the results of this study.

Material and methods

Sampling

VWA inspectors sampled 292 Asian THPs for oral use on the Dutch market between December 2004 and June 2007. The aim of the sampling plan was to collect commercially available Asian THPs that could potentially contain significant amounts of mercury, arsenic or lead. Samples were of Ayurvedic, Chinese and Tibetan origin and were directly collected from importers, producers and vendors throughout the Netherlands. The locations were selected from the VWA inspection database or identified by an Internet search. No THPs were purchased via Internet.

Per product, one or more units of at least 20 g were sampled. Criteria for inclusion were an apparent relation to Ayurveda, TCM or other traditional Asian medicine systems and the presence of ingredients used traditionally in these medicine systems. THPs lacking such features were excluded. For instance, a food supplement made in China with the contemporary ingredient spirulina or an American supplement with the traditional ingredient gingseng but lacking a link with TCM, would be excluded.

Sampling was aimed at multi ingredient THPs with herbs and minerals but some single herb preparations were also included. The labeling of each potential sample was scanned for ingredients listed in Table 1 reported to contain mercury, arsenic or lead [7, 16, 34, 35, 42, 44, 48]. We also compared the product name to a list of names of classical formulas for Ayurvedic THPs (Table 2) and a list of classical formulas for Chinese THPs (Table 3) that are reported to include mercury, arsenic or lead compounds. Table 2 was compiled from the official Ayurvedic Formulary published by the Indian government [34, 35] by selecting from this source the names of formulas for THPs for oral use that included ingredients with lead, mercury or arsenic.

Table 3 was compiled from the Pharmacopoeia of the People's Republic of China (English edition of 1997) by the British Medicine Control Agency supplemented with names of formulas for Chinese THPs which were described in a TCM handbook to contain ingredients with lead, mercury and arsenic [40, 48]. Also included in Table 3 were Chinese THPs reported to contain mercury, arsenic or lead [5, 10, 16, 25, 33, 44]. THPs with ingredients listed in Table 1 or product names listed in Tables 2 or 3 were sampled preferentially, but other THPs were sampled as well.

Table 1 Traditional names of lead, mercury and arsenic preparations used as ingredients of Asian THPs

Element	Pharmaceutical or traditional names of preparation	Synonyms and related preparations or compounds
Lead	Lithargyrum (lead monoxide)	A: girisindura, litharge, mrddara srnga, mrddarasrnga, muddarasankha, sindura C: mi tuo seng
	A: Naga bhasma	A: ahi, bhujagalauha, sisaka,
Mercury	A: Parada (purified mercury)	A: chapala, haraja; isa, mrtasuta, rasa, rasa sindura, rasaraja rasendra, rasesa, rasesvara, rasottama, suddhasuta, suta, sutaka,
	Cinnabar (red mercury sulfide)	A: aruna, caliya, hingula, hingulotthaparada, hingula sambhava suta, suddha hingula C: zhu sha, chen sha, dan sha
	Calomel (mercurous chloride) Hydrargyri oxydum rubrum (red mercury oxide)	C: qing fen, shui yin fen, gong fen, chen fen
Arsenic	Orpiment (arsenic trisulfide)	A: ala, haratala, haritala, tala, talaka C: cihuang
	Realgar (arsenic sulfide or arsenic disulfide)	A: manahsila, manayola, sila, C: xiong huang, xiong jing, yao huang
	Arsenolite (white arsenic or arsenic trioxide)	A: gauripasana, malla, svetamalla C: pishi

A: traditional name used in Ayurveda

C: traditional name used in TCM

References: [7, 16, 34, 35, 42, 44, 48]

Table 2 Formulas for Ayurvedic THPs for oral use possibly containing mercury, arsenic or lead

Ayurvedic THP	Synonyms
Agnitundivati	Angitundi Vati
Ajirna Kantaka Rasa	
Amavatari Rasa	Amavatari Ras; Amvatari Ras
Amlapittantaka Rasa	Amlapittantka Rasa
Anandabhairava Rasa	Anand Bhairav Ras; Anandbhairav Ras
Arogyavardhini Gutika	
Arsakuthara Rasa	Arsh Kuthar Ras; Arsha Kuthar Rasa
Astaksari Gutika	
Asvakancuki Rasa	Ashwakanchuki Ras
Balarka Rasa	Balark Rasa
Bola Parpati	Bol Parpati
Brhadagnikumara Rasa	
Brhanmaricadya Taila	
Brhat Candrodaya Makaradhvaja	
Brhat Garbhacintamani Rasa	Brihat Garbhachintamani Rasa; Garbha Chintamani Ras Vrihat
Brhat Kasturibhairava Rasa	Brihat Kasturi Bhairav Rasa
Brhat Nrpavallabha Rasa	
Brhat Purnacandra Rasa	Brihat Purnachandra Rasa
Brhat Sarvajvarahara Lauha	Sarvajwarhar Loha Br.
Brhat Vangesvara Rasa	Bangeshwar Ras Brihat
Brhat Vatacintamani Rasa	Brihat Vata Chintamani; Brihat Vatchintamani Rasa
Brhat Vidyadharabhra Rasa	
Brhat Visamajvarantaka Lauha	
Brhatcchrngarabhra Rasa	
Candrakala Rasa	Chandrakala Ras (Tamra Yukta)

Table 2 (Continued)

Ayurvedic THP	Synonyms
Candramrta Rasa	Chandramrit Ras
Candramsu Rasa	
Caturbhuja Rasa	Chaturbhuj Rasa
Caturmukha Rasa	Chaturmukh Ras
Cintamanicaturmukha Rasa	
Ekangavira Rasa	Kangveer Ras; Ekangvir Ras
Gaganasundara Rasa	
Gandamala Kandana Rasa	
Gandhaka Vati	Gandhak Bati (Raj Bati)
Garbha Cintamani Rasa	
Garbhapala Rasa	Garbhapal Ras
Gorocanadi Vati	Gorochandi Vati
Grahani Kapata Rasa	Grahani Kapat Ras
Guducyadi Modaka	
Gulma Kalanala Rasa	Gulma Kalanal Ras
Haritala Bhasma	Harital Bhasma
Hemagarbha Pottali Rasa	
Hemanatha Rasa	Hemnath Ras
Hingulesvara Rasa	
Hiranya Garbha Pottali Rasa	
Hrdanyarnava Rasa	Hridayarnava Ras
Icchabhedi Rasa	Ichhabhedi Ras (Jaypal Yukta)
Jalodarari Rasa	
Jvaraghni Gutika	
Jvarankusa Rasa (Ka)	
Jvararyabhra	
Kalakuta Rasa	
Kanakasundara Rasa	
Kancanabhra Rasa	
Kantavallabha Rasa	Khantavallabha Rasa
Karpura Rasa	Karpur Ras
Kasisadi Ghrta	
Kasturi Bhairava Rasa	Kastoori Bhairav Ras
Kasturyadi Vayu Gutika	

Table 2 (Continued)

Ayurvedic THP	Synonyms
Kravyada Rasa	
Krmi Kuthara Rasa	Krimi Kuthar Rasa
Krmimudgara Rasa	Krimimudgar Ras
Kumarakalyana Rasa	Kumarkalyan Rasa
Laghvananda Rasa	
Lakanatha Rasa	
Laksminarayana Rasa	Laxminarayan Rasa
Laksmivilasa Rasa	Laxmivilas Ras; Laxmi Vilas Rasa
Lauha Parpati	
Lila Vilasa Rasa	Lilavilas Rasa
Mahagandhaka Vati	
Maha Jvarankusa Rasa	Mahajwarankush Ras; Mahajvarankush Rasa
Maha Laksmivilasa Rasa	Mahalaxmi Vilas Rasa; Mahalaxmivilas Ras; Mahalakshmi Vilas Ras
Mahatarunarka Rasa	
Maha Vata Vidhvamsana Rasa	Mahavatvidhvasan Ras; Mahavatvidhvansan Rasa; Mahavat Vidhwans Ras
Maha Vatagajankus A Rasa	
Maha Yogaraja Guggulu	Maha Yograj Guggul
Makara Dhvaja	Makardhwaj; Makardhvaj Rasa
Mallasindura	Mallasindur
Manikya Rasa	
Manmathabhra Rasa	
Maricadya Taila	
Mrtyunjaya Rasa	Mrityunjai Ras
Muktapancamrta Rasa	Mukta Panchamrit Ras; Moti Yukt
Naga Bhasma	Nag Bhasma
Naraca Rasa	
Navaratnarajamrganka Rasa	
Nidrodaya Rasa	

Table 2 (Continued)

Ayurvedic THP	Synonyms
Nityananda Rasa	
Nrpativallabha Rasa	Nripatiballabha Ras
Pancamrta Lauha Guggulu	
Pancamrta Parpati	Panchmrita Parpati
Pancanana Rasa	
Piyusavalli Rasa	Piyushvali Ras
Prabhakara Rasa	
Pradarantaka Lauha	Pradarantak Lauh
Pradarantaka Rasa	Pradarantak Rasa
Pratapalankesvara Rasa	Prataplankeshwar
Purnacandra Rasa	Purnachandra Ras
Puspadhanva Rasa	Pushpadhanwa
Putapakva Visama	
Jvarantaka Lauha	
Rajamrganka Rasa	
Rajata Bhasma	
Rasa Parpati	Ras Parpati
Rasakarpura	
Rasamanikya	Rasa Manikya
Rasapuspa	
Rasaraja Rasa	Ras Raj Ras; Rasraj Rasa
Rasasindura	Rasa Sindur
Ratnagiri Rasa	
Samirapannaga Rasa	Samirpannag Rasa
Sankha Vati	Shankhavati; Shankh Vati
Saubhagya Vati	Saubhagya Bati
Siddhapranes Vara Rasa	Siddhapraneshwar Rasa
Sirah Suladi Vajra Rasa	Shirahsuladrivajara Ras
Smrti Sagara Rasa	Smritisagar Ras; Smriti Sagar Rasa
Sri Jayamangala Rasa	
Sri Ramabana Rasa	
Srinrpativallabha Rasa	
Srngarabhra Rasa	Shringarabhra Ras
Sucikabharana Rasa	
Sukramatrka Vati	Shukramatrika Vati
Sulavajrini Vatika	Shoolvarjini Vati

Table 2 (Continued)

Ayurvedic THP	Synonyms
Sutasekhara Rasa	Soothashekara; Sutshekhar Rasa; Sutsjekhar Rasa
Sutikabharana Rasa	Sutikabharan Ras
Svacchanda Bhairava Rasa	
Svalpanayika Curna	
Svarna Bhasma	Swarna Bhasma
Svarna Parpati	Swarna Parpati
Svarnabhupati Rasa	
Svarnasindura	
Svarnavanga	
Svasa Kasa Cintamani Rasa	Shawsa Kasa Chintamani; Swasakas Chintamani Rasa
Svasakuthara Rasa	Swas Kuthar Ras; Swasa Kuthara Ras; Shwas Kuthar Rasa
Tamra Bhasma	
Tamra Parpati	
Tarakesvara Rasa	
Tarunarka Rasa	
Tribhuvanakirti Rasa	Tribhuvankirti
Trivanga Bhasma	Tribanga Bhasma
Trivikrama Rasa	
Unmadagajakesari Rasa	
Vajrakapata Rasa	
Vanga Bhasma	
Varisosana Rasa	
Varja Bhasma	
Vasanta Malati Rasa	
Vasanta Tilaka Rasa	
Vasantakusumakara Rasa	Vasant Kusumakar Rasa
Vata Gajankusa Rasa	Vatagajankush Ras; Vatgajankush Rasa
Vatagnikumara Rasa	
Vatakulantaka Rasa	Vatkulantak Ras; Vatakulantak Ras
Vataraktantaka Rasa	
Vatari Rasa	Vatari Ras
Vatavidhavamsana Rasa	
Vidanga Lauha	
Yakrdari Lauha	
Yakrtplihari Lauha	Yakritplihari Loha
Yogendra Rasa	

References: [34, 35]

ICP-MS determination of metals and metalloids

A routine method for the determination of metals and metalloids in herbal preparations with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was developed and validated for several elements including arsenic, lead, and mercury. Routinely cadmium, zinc, copper, nickel, chromium and aluminum were monitored as well. Samples were completely homogenized by grinding them to powder with a grinder (Retsch). To 20 - 100 mg (depending on the elements to be measured) of the powdered sample 3 ml nitric acid (HNO_3, 65%) and 1 ml hydrogen peroxide (H_2O_2, 30%) were added. A destruction step was then performed using a microwave oven (Ethos Plus Microwave Labstation, Milestone) in which the samples were treated for 25 minutes at 200ºC. The solution produced by the destruction process was then quantitatively transferred with de-ionized water from the destruction vessel to a 50 ml volumetric flask. Before filling the flask to the 50 ml mark with de-ionized water, 500 µl aureumchloride (AuCl, 100 mg/L) solution was added. Throughout the analysis Milli-Q grade de-ionized water was used. The sample solutions were diluted (up to 10000 times) when necessary to fit within the calibration curve and measured with ICP-MS (Thermo Electron PQ Excell) 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 [49].

In order to test if each destruction vessel was clean prior to use, test runs were performed on each vessel with only 3 ml HNO_3 (65%) and 1 ml H_2O_2 (30%) before each series of samples. This was repeated after each series in order to confirm that no metals or metalloids were left in the vessels. For the in house validation of the method, first the limits of detection and quantification (LOD and LOQ) were determined. The LOD was defined as three times the residual standard deviation of the standard calibration curve. The LOQ was defined as twice the LOD. The range of the calibration curve was used to calculate the range of application. Samples were diluted and reanalyzed when concentrations appeared to exceed the highest concentration of the calibration curve. The accuracy of the method was determined by analysis of a Certified Reference Material (CRM)(IRMM; BCR 482: trace elements in Lichen). The analytes of this CRM had to be within the 95% confidence interval of the certified values.

Furthermore, the relative standard deviation (RSD_r) was measured by replicate analysis (n=6) of the CRM under repeatability conditions. The RSD_r is a measure of the degree of agreement of results obtained by the same analyst with identical reagents, equipment and instruments within a short period of time under conditions kept as constant as possible. The CRM was also used as quality control sample in each series of measurements. The expanded measurement uncertainty (in mg/kg) was determined as the reproducibility from measurements (n>10) of this CRM, analyzed by different persons on different days.

Table 3 Formulas for Chinese THPs for oral use possibly containing mercury, arsenic or lead

Product Name	Toxic substance
Ailing Yihao [a]	Arsenolite
Angong Niuhuang San, -Wan [b, c, d]	Cinnabar, Realgar
An Shen Bu Nao Pian (Ansenpunaw tablets) [c]	Cinnabar
Bai Zi Yang Xin Wan [c]	Cinnabar
Baochi San	Cinnabar
Baochiwanying-san [e]	Cinnabar
Baolong Wan	Cinnabar
Bao Ning Dan [f]	
Bao Ying Dan [c]	Cinnabar
Bingpen San	Cinnabar
Biwen San	Cinnabar
Bushen Yinao Pian	Cinnabar
Chunyang Zhengqi Wan	Cinnabar, Realgar (+ *Aristolochia* Radix)
Ci Zhu Wan [b, c]	Cinnabar
Da Huo Luo Wan [d]	high mercury content reported [d]
Da qili San	Cinnabar [g]
Dendrobium Moniliforme Night Sight Pills [d]	high mercury content reported [d]
Ding Xian Wan [b]	Cinnabar
Ershiwuwei Shanhu Wan	Cinnabar
Ershiwuwei Songshi Wan	Cinnabar
Feierwanyaopian [e]	
Geng Yi Wan [b]	Cinnabar
Hongling San	Cinnabar, Realgar
Hua Chong Wan	Red lead oxide
Hui Chun Dan [b]	Cinnabar
Hu Po Bao Long Wan (Po Lung Yuen Med. Pills) [c]	Cinnabar
Jian Nao Wan (healthy brain pills) [c]	Cinnabar
Jierezhenluesan [e]	Cinnabar
Jiusheng San	Calomel, Hydrargyri Oxydum Rubrum
Jiuyi San	Hydrargyri Oxydum Rubrum
Jufangniuhuang-qingxinwan [e]	Cinnabar, Realgar
Jufang Zhibao San	Cinnabar, Realgar
Liushenwan[e]/ Liu Shen Wan [b]	Realgar
Meihua Dianshe Wan	Cinnabar, Realgar
Niuhuang Baolong Wan	Cinnabar, Realgar
Niu Huang Cheng Qi Tang [b]	Cinnabar, Realgar
Niu Huang Chiang Ya Wan	high mercury and arsenic contents reported [d]
Niu Huang Ching Hsin Wan	high mercury and arsenic contents reported [d]
Niuhuang Jiedu Pian	Realgar
Niuhuang Jiedu Wan	Realgar
Niuhuang Qianjin San	Cinnabar
Niuhuangqingfei-san [e]	Cinnabar, Realgar
Niu Huang Qing Xin Wan [b]	Cinnabar, Realgar
Niuhuang Zhenjing Wan	Cinnabar, Realgar
Peaceful [c]	Cinnabar
Pinggan Shuluo Wan	Cinnabar
Po Lung Yuen Med. Pills [c]	Cinnabar
Qianjinsan [e]	Cinnabar
Qi Li San [b, c]	Cinnabar
Qizhen Wan	Cinnabar, Realgar
Renqing Changjue	Cinnabar
Renqing Mangjue	Cinnabar
San Li Hui Chun Dan [c]	Cinnabar
Shayao	Cinnabar, Realgar
She Dan Chen Pi San [c]	Cinnabar
Sheng Tie Luo Yin	
Shixiang Fansheng Wan	Cinnabar (+ A*ristolochia* Radix)
Shugan Wan	Cinnabar
Shuzheng Pian	Cinnabar, Realgar
Suhexiang Wan/ Su He Xian Wan [b, c]	Realgar
Ta Huo Lo Tan [d]	high mercury and arsenic contents reported [d]

Table 3 (continued)

Product Name	Toxic substance
Tianwang Buxin Wan	Cinnabar
Tiewadan [e]	Cinnabar
Tse Koo Choy [c]	Calomel
Tsai Tsao Wan [d]	high mercury and arsenic contents reported [d]
Tzuhsueh Tan (Zi Xue Dan) [c]	Cinnabar
Wanshi Niuhuang Qingxin Wan/ Wan Shi Niu Huang Qing Xin Wan [c]	Cinnabar
Watermelon frost (Xi Gua Shuang) [c]	Cinnabar
Xiangsu Zhengwei Wan	Cinnabar
Xiao'er Baishou Wan	Cinnabar
Xiao'er Huadu San	Realgar
Xiao'er Jindan Pian	Cinnabar (+ *Aristolochia manshuriensis)*
Xiao'er Jingfeng San	Cinnabar, Realgar
Xiaoerqifengsan	Cinnabar
Xiao'er Qingre Pian	Cinnabar, Realgar
Xiaoerqiyingwan [e]	Cinnabar (arsenic also detected) [e]
Xiao'er Zhibao Wan	Cinnabar, Realgar
Xi Gua Shuang (Watermelon frost)	Cinnabar
Yatong Yili Wan	Cinnabar, Realgar
Yinianjin	Cinnabar
Yingerbaofeining [e]	Cinnabar
Yingerle [e]	Cinnabar
Yixian Wan	Cinnabar, Realgar
Yi Yuan San [b]	Cinnabar
Zaizao Wan	Cinnabar
Zhi Bao Dan	Cinnabar, Realgar
Zhou Che Wang [b]	Calomel
Zhuhuang Chuihou San	Realgar
Zhu Sha An Shen Wan [b, c] (Cinnabar sedative pill)	Cinnabar
Zijin Ding/ Zi Jin Ding [c]	Cinnabar, Realgar
Zixue	Cinnabar
Zi Xue Dan [b,c] (Tzuhsueh Tan) [c]	Cinnabar

Based on [48]. Additional references: [a] [44]; [b] [40]; [c] [16]; [d] [5]; [e] [10]; [f] [25]; [g] [33]

Table 4 lists the method performance characteristics as the LOD, LOQ, range of application, accuracy, RSD_r and the measurement uncertainty (in mg/kg). According to the AOAC guidelines for single laboratory validation of chemical methods for botanicals and dietary supplements, the accuracy has to be between 75 and 120% at the measured concentrations [50]. The values for the accuracy of the method for mercury, arsenic and lead as listed in Table 4 fitted well within these limits as did the other elements that can be analyzed with this method. The RSD_r for all elements was found to be below 10% with mercury displaying the highest value of 9.5%. These values are also in accordance with this AOAC program that requires the RSD_r to be below 10% at the measured concentrations. For the elements Al, Ni, Cu and Zn the limits of detection and quantification were about 10 times higher than that of mercury, which has an LOD of 0.03 mg/kg. Addition of AuCl to the sample and flush solutions helped to overcome initial problems with persistent mercury residues in the system because of the capacity of mercury to complex with gold [51].

Table 4 Characteristics (limit of detection (LOD), limit of quantification (LOQ), range of application, accuracy and expanded measurement uncertainty) of the analytical method for Pb, As and Hg determined by in house validation

Element	LOD (mg/kg)	LOQ (mg/kg)	Range of application (mg/kg)	Accuracy [a] (%)	RSD_r [b] (%)	Expanded Measurement Uncertainty [c] (mg/kg)
Pb	0.18	0.36	0.25 – 12.5	103	5.1	3.4 (at level of 37.3 mg/kg)
Hg	0.03	0.06	0.25 – 12.5	99	9.5	0.047 (at level of 0.549 mg/kg)
As	0.06	0.13	0.25 – 12.5	96	2.1	0.09 (at level of 0.89 mg/kg)

[a] Accuracy has to be between 75 and 120% [50]

[b] Relative Standard Deviation (RSDr) may not exceed 10% [50]

[c] The expanded measurement uncertainty (mg/kg) was calculated as the reproducibility at the concentration level of the CRM.

Estimation of the weekly mercury, arsenic or lead intake and selection of safety limits

At the time of completion of this study neither Dutch nor European food law contained maximum levels for mercury, arsenic or lead in food supplements. We based our enforcement actions therefore on the legal requirement that only safe foods are to be placed on the market. In order to assess the safety of a THP we estimated the associated intake of mercury, lead and arsenic and subsequently compared the estimated intakes of these elements to established safety limits. The estimated mercury, arsenic or lead intake per week was calculated from the analytically determined concentrations of these elements, the unit dose weight and the highest recommended daily dose stated on the label of the THP. In a few cases where no recommended dose could be identified on the label or from other sources a default value was used of, depending on the dose form, one pill or 1 g of powder per day. We did not consider background exposure from other sources such as foods, water or ambient air in our risk assessment. For each of these three elements we selected an established safety limit by reviewing risk assessments by international and national bodies. We preferentially considered safety limits

for inorganic mercury, arsenic and lead compounds because available sources on the speciation of these elements in Chinese, Ayurvedic and other Asian THPs consistently indicated that mercury, arsenic and lead are present as inorganic compounds [2, 4, 10, 16, 17, 34, 35, 37, 38, 40-43, 45, 52, 53]. Examples of the risk assessments by international and national bodies reviewed are shown in Table 5 [9, 54-61].

Table 5 Safety limits for lead, mercury and arsenic

Element	Safety limit	Safety limit set by	Safety limit set for	Tolerable weekly intake (mg/week) [a]
Lead	4.5 µg/day	US Pharmacopoeia-specification [b]	Lead from a daily dose of calcium carbonate	0.0315
	25 µg/kg bw/week	JECFA-PTWI [c]	Lead from all food sources	1.5
Mercury	0.3 µg/kg bw/day	US EPA-RfD [d]	Mercuric chloride	0.126
	5 µg/kg bw/week	JECFA-PTWI [e]	Total mercury from diets high in methylmercury	0.3
	2 µg/kg bw/day	IPCS-TDI [f] /RIVM-TDI [g]	Inorganic mercury	0.84
Arsenic	0.3 µg/kg bw/day	US EPA-RfD [h]	Inorganic arsenic	0.126
	1.0 µg/kg bw/day	RIVM-TDI [g]	Inorganic arsenic	0.42
	15 µg/kg bw/week	JECFA-PTWI [i]	Inorganic arsenic	0.90
	3 µg/kg bw/day	ANZFA-TDI [j]	Inorganic arsenic	1.26

[a] Calculated for a 60 kg adult

References: [b] [9]; [c] [54]; [d] [55]; [e] [56]; [f] [57]; [g] [58]; [h] [59]; [i] [60]; [j] [61]

From the selected safety limit we calculated a tolerable weekly intake (TWI) limit for a 60 kg adult. For mercury we selected the TDI for inorganic mercury of 2 µg/kg bw/day set independently by the Dutch National Institute for Public Health and the Environment (RIVM) and the International Program on Chemical Safety (IPCS) [57, 58]. This TDI is equivalent to a TWI limit of 0.84 mg per week for a 60 kg adult (Table 5). For arsenic we selected the TDI for inorganic arsenic set by RIVM of 1.0 µg/kg bw/day, equivalent to a TWI limit of 0.42 mg per week for a 60 kg adult (Table 5) [58]. For lead we selected the provisional tolerable weekly intake (PTWI) limit for lead from all sources of 25 µg/kg bw/week established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), equivalent to a TWI limit of 1.5 mg per week for a 60 kg adult (Table 5) [54, 62].

In agreement with European Regulation (EC) No. 333/2007 we established for enforcement purposes for mercury, arsenic or lead each, the estimated weekly intake level at which the TWI limit is exceeded beyond reasonable doubt [63]. We derived these VWA action limits by adding to the selected TWI limit for the element, the analytically obtained expanded measurement uncertainty for the estimated weekly intake (in mg/week). For the estimated lead intake of a THP, a VWA action limit of 1.85 mg per week was derived. The VWA action limit for the estimated weekly intake of mercury was 1.31 mg per week and for arsenic the VWA action limit was a

maximum estimated weekly intake of 0.510 mg per week. Recently the European Commission established for food supplements maximum product levels of 0.10 mg/kg for mercury and 3.0 mg/kg for lead, which will enter into force on the 1st of July 2009 [64]. No limit has yet been proposed for arsenic in food supplements. We also applied these EU maximum levels to our data to estimate the effect of this new legislation on the availability of Asian THPs with mercury or lead on the Dutch market. Furthermore, we applied safety limits used in several pertinent studies on mercury, arsenic or lead in Asian THPs to our data in order to explore how the selection of other relevant limits would have affected the outcome of the study.

Results

Mercury, arsenic or lead contents of THPs

In total 186 (64%) of 292 THPs used in Ayurveda, TCM and TTM contained arsenic, mercury or lead. Figure 1 shows for lead, mercury and arsenic separately, how the THPs were distributed over increasing concentration ranges of these elements. The labeling of less than 20% of the THPs did contain indications that matched an entry of Tables 1, 2 or 3, which would suggest the presence of ingredients with mercury, arsenic or lead levels. The remaining THPs were sampled according the general criteria of the defined sampling protocol to test the presence of mercury, arsenic or lead in Asian THPs prevalent on the Dutch market.

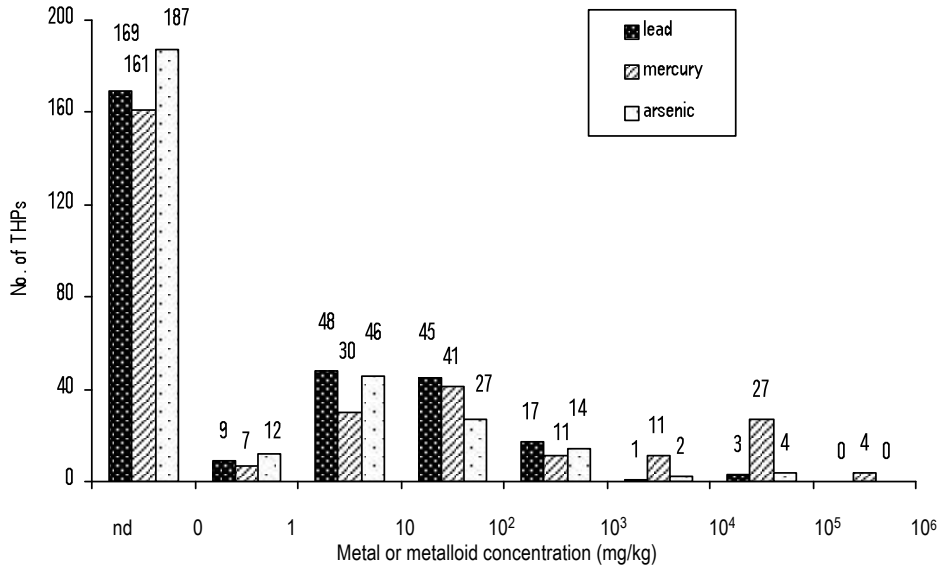


Figure 1 Concentrations of lead, mercury and arsenic in THPs; nd: not detected

Lead was present in 123 (42%) THPs and levels ranged from 0.5 to a maximum of 60 000 mg/kg that was found in the Ayurvedic THP Vasant Yog. The average lead level in the positive samples was 1,000 mg/kg; the median level was 13 mg/kg and the 95th percentile 573 mg/kg. Mercury was found in 131 (45%) THPs in levels from 0.2 up to 171 000 mg/kg and the highest concentration was found in the Ayurvedic THP Yogendra Ras. The average mercury level in the positive samples was 11 800 mg/kg, the median level was 50 mg/kg and the 95th percentile was 86 000 mg/kg. In 105 (36 %) of the 292 collected THPs arsenic was present in levels ranging from 0.2 up to 89 800 mg/kg, the latter in the Ayurvedic THP Swashanti Yog. The average arsenic level in the positive samples was 2300 mg/kg; the median level was 7.6 mg/kg and the 95th percentile for arsenic of 7600 mg/kg. In a few cases low levels of cadmium were found but the associated estimated intakes were not above international safety limits (data not shown). Table 6 shows for lead, arsenic and mercury separately the number of Ayurvedic, Chinese and Tibetan THPs in which these elements were detected above the LOQ.

Table 6 Number of Ayurvedic, Chinese and Tibetan THPs with lead, mercury or arsenic above the limit of quantification

	No. of Ayurvedic THPs	No. of Chinese THPs	No. of Tibetan THPs
Total of the THP type sampled	242	42	8
Lead above LOQ [(a)]	105	13	5
Mercury above LOQ	117	8	6
Arsenic above LOQ	85	15	5
Total of THPs with at least one element above LOQ	161	19	7

[a)] LOQ: Limit of quantification

Estimated weekly metal or metalloid intakes in relation to the selected safety limits

In total 64 (22%) of 292 THPs were likely to result in weekly intakes of lead, arsenic or mercury, separately or in combination, above the selected TWI limits when used according to recommendations. Levels of these elements in 59 of these 64 THPs would result in intakes that also exceeded the VWA action limits; this signified that in total for 59 (20%) of the 292 THPs the estimated intakes of lead, arsenic or mercury would exceed the selected TWI limits beyond reasonable doubt (Table 7). In total 25 THPs from Table 7 carried indications that according to Table 1, 2 and 3 suggested that ingredients high in mercury, arsenic or lead could be present. Use according recommendations of the Ayurvedic THPs 'Raja Parvatini', 'Vasant Yog' and 'Nature Slim' would result in daily doses of lead, mercury and arsenic above all 3 VWA action limits simultaneously.

Table 7 Asian THPs resulting in estimated lead, mercury or arsenic intakes significantly above one or more TWI limits

Asian THP	Type of Asian THP [(a)]	Arsenic	Mercury	Lead
		mg/week		
Abhrak Bashma	A	**0.82**	nd	1.3
Abhrak Bhasma	A	**1.0**	nd	*1.6*
Agni Vati	A	nd	**72**	nd
Amrit Prash	A	0.1	**5.1**	0.04
Amrit Ras	A	**1.8**	**377**	0.27
Arjuna Hepatone	A	nd	**198**	1,0
Arogya Vardhini Vati	A	nd	**338**	nd
Arogyavardhini Vishisht	A	0.04	**1.9**	0.21
Ayfer	A	0.05	0.12	**4.0**
Ayu-Hirakam	A	0.21	**2.6**	0.14
Bat Chintamani	A	**2.2**	**471**	0.26
Bo Zi Yang Xin Wan	C	nd	**1473**	nd
Chandraprabha	A	0.07	nd	**15**
Diabetomed	A	**1.4**	**3.5**	0.41
Ding Xin Wan	C	*0.44*	**430**	1.3
Ere Forte	A	nd	**12**	0.34
Femi-Smilin	A	**7.5**	0.73	0.33
Gandhadi Yog	A	nd	**11**	nd
Ghandramsu Ras	A	nd	**132**	0.14
Guggulu	A	0.38	**7.9**	**3.1**
Gurgum-8	T	**2.9**	**317**	0.04
Guyu Depak	T	*0.47*	**192**	0.1
Guyu Depak	T	**0.94**	**239**	0.15
Heartina	A	**262**	**340**	nd
Herba Figura	A	0.07	**2.3**	0.07
Herbo Gastrol	A	nd	**14**	0.33
Herbogastrol	A	nd	**12**	0.23
Herbogastrol	A	nd	**27**	nd
Hridaya Chintamani	A	**2.6**	**396**	nd
Jeevani Vati	A	0.22	**173**	nd
Kamachudamani Ras	A	**7.7**	**22**	0.27
Liv.52	A	nd	nd	**2.6**
Luxmi Vilas Classic	A	0.02	**138**	0.05
Maha Manjishtadi Ghan Vati	A	0.04	**4.2**	0.09
Maha Ras Guggul	A	**1.0**	*1.2*	0.03
Mahabatbiddhonsan Ras	A	0.08	**69**	**78**

Table 7 (continued)

Asian THP	Type of Asian THP [a]	Arsenic	Mercury	Lead
		mg/week		
Makar Wazra	A	**5.0**	**903**	nd
Mutik-25	T	**0.53**	**146**	nd
Natureslim	A	**79**	**111**	**192**
Pesin	A	**0.9**	**923**	0.07
Raja Parvartini	A	**2.2**	**7.2**	**5.4**
Ras Yog	A	0.04	**92**	nd
Ru Pi Xiao Pian	C	**1.1**	nd	nd
Sanjiwani	A	**427**	nd	nd
Shatavari	A	**1.3**	**2.4**	0.14
Shingorabrak	A	nd	**162**	nd
Shringrabhra	A	**0.8**	**20**	0.12
Shringrabhra	A	nd	**26**	1.2
Sirshool Vajraras	A	0.05	**299**	1.1
Swas Kuthar Ras	A	**66**	**65**	0.11
Swashanti Yog	A	**200**	**118**	nd
Sworna Yog	A	**1.5**	**70**	nd
Tianwang Buxin Wan [b]	C	nd	**24**	0.17
Triyog Misran	A	nd	**71**	nd
Ulceromed	A	0.03	**22**	0.10
Vasant Yog	A	**0.60**	**57**	**105**
Yogendra Ras	A	0.06	**274**	0.18
Yograj Guggulu	A	0.08	**1.4**	*1.6*
Zhui Feng Tou Gu	C	**5.1**	**1747**	0.67

Estimated element intake values in bold are above the VWA action limit for that element at the proposed use level; values in italics are between the TWI limit and the VWA action limit.

nd: not detected.

[a] THP types sampled: 'A' for Ayurvedic THPs, 'C' for Chinese THPs, 'T' for Tibetan THPs

[b] The daily dose assumed to be 1 pill (0.195 g)

Figure 2 shows for lead, mercury, and arsenic, respectively, the distribution of THPs over increasing ranges of weekly intake estimates in relation to the individual TWI limits for these elements. The median intake of lead with THPs positive for this metal was 0.11 mg/week, the median intake of mercury with THPs containing this metal was 0.52 mg/week and the median arsenic intake with THPs positive for this metalloid was 0.08 mg/week. The lead intake estimates at proposed dose levels for 10 THPs exceeded the TWI limit of 1.5 mg lead per week (range 1.6-192 mg/week) (Figure 2) and the lead intake estimates for 8 of these also exceeded the VWA action limit of 1.85 mg/week. Of these 10 THPs, 8 THPs would at their proposed dose levels also result in intakes above TWI limits for arsenic or mercury or both as well.

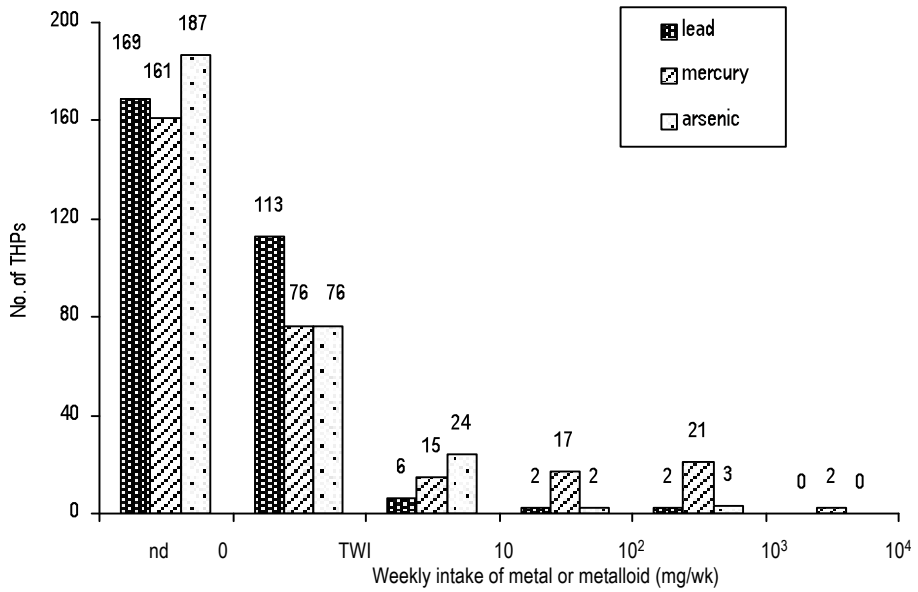


Figure 2 Estimated weekly intake of lead, mercury and arsenic resulting from intake of THPs at their proposed use levels; 'TWI' refers to the tolerable weekly intake limit for each element; for lead the TWI is 1.5 mg/week, for mercury 0.84 mg/week and for arsenic 0.42 mg/week; nd: not detected

The estimated mercury intake at the proposed dose level of 55 THPs would be above the TWI limit of 0.84 mg inorganic mercury per week (range 0.92-1747 mg/week) (Figure 2) and mercury intake estimates of 50 of these were also above the VWA action limit of 1.31 mg/week. Of 26 of these 55 THPs, the intake of arsenic or lead resulting from their proposed dose levels, would also be in excess of the TWI limits for one or both of these two elements. We found 29 THPs where the proposed dose levels would result in arsenic intakes above the TWI limit for arsenic (range 0.44-427 mg/week) (Figure 2) and the proposed dose levels of 26 of these would result in intakes above the VWA action limit for arsenic of 0.510 mg/week. Additionally, the proposed dose levels of 24 of these 29 THPs would also give rise to mercury or lead intakes in excess of the TWI limits selected for these metals.

Table 8 shows for lead, mercury and arsenic separately, the number of Ayurvedic, Chinese THPs and Tibetan THPs for which use at their proposed dose levels would result in intakes above TWI limits for each of these elements.

Table 8 Number of Ayurvedic, Chinese and Tibetan THPs where use at the proposed dose levels results in lead, mercury or arsenic intakes above TWI limits

	No. of Ayurvedic THPs	No. of Chinese THPs	No. of Tibetan THPs
Total of THP in study	242	42	8
Lead intake above TWI	10	na	na
Mercury intake above TWI	46	5	4
Arsenic intake above TWI	22	3	4
Total of THPs with at least one element above TWI	50	5	4

na: not applicable

In addition to the TWI limits applied above, we also compared our estimated intake data to other relevant safety limits for mercury, arsenic and lead as well as to the maximum levels for lead and mercury in food supplements recently established by the European Commission [64]. Table 9 shows the number of THPs where use according to recommendations would result in intakes of mercury, arsenic and lead above these other safety limits [9, 54-61, 64]. When the newly defined European maximum levels for lead and mercury were applied, the highest number of exceedances was seen: 101 (82%) of 123 of THPs positive for lead and 130 (99%) of 131 of the products containing mercury were above these limits (Table 9).

Discussion

Mercury, lead and arsenic were detected in 186 (64%) of 292 THPs used in Ayurveda, TCM and TTM sampled in the Netherlands. Use at the proposed dose levels of 59 (20%) of these 292 THPs would result in estimated mercury, arsenic and lead intakes significantly above established safety limits. Furthermore, 82% of the THPs with lead and 99% of the THPs containing mercury exceeded the recently established European maximum product levels for food supplements (Table 9) [64]. Our results are in good agreement with several studies on mercury, arsenic and lead contents of Asian THPs sampled in Western and Asian countries. Saper et al. reported in 2004, mercury, arsenic or lead in 20% of 70 Ayurvedic THPs collected in Boston and the estimated intakes of these elements at proposed use levels were all above selected safety limits [9]. Lead was found in 13 THPs (19%), mercury and arsenic both in 6 preparations (9%). Saper et al. used a US Pharmacopoeia specification as a safety limit for lead that was considerably more conservative than the JECFA PTWI for lead [54] used in our study which would explain why we found a lower percentage of THPs with estimated lead intakes above the TWI limit (Tables 5, 8 and 9).

Table 9 Lead, mercury and arsenic intakes with THPs compared to various safety limits for these elements and EU maximum product levels for lead and mercury

Element	No. of THPs positive for element	No. of THPs with estimated element intakes or element level above limit [a]	Risk assessment or risk management body responsible for limit and type of limit	Limit recently applied for Asian THPs in:
Lead	123	10	JECFA-PTWI [b]	this study; [43]
		99	US Pharmacopoeia-specification [c]	[9]
		101	EU-maximum level [d]	this study
Mercury	131	55	RIVM-TDI [e]/ IPCS-TDI [f]	this study
		68	JECFA-PTWI [g]	[43]
		91	US EPA-RfD [h]	[9]
		130	EU- maximum level [d]	this study
Arsenic	105	29	RIVM-TDI [e]	this study
		17	ANZFA-TDI [i]	[43]
		21	JECFA-PTWI [j]	[10]
		39	US EPA- RfD [k]	[9]

[a] Calculated either by comparing the estimated intake of a element resulting from the recommended use of the THP with the safety limit referred to in the next column calculated for a 60 kg adult or by comparing a maximum product level to the element concentration.

References: [b] [54]; [c] [9]; [d] [64]; [e] [58]; [f] [57]; [g] [56]; [h] [55]; [i] [61]; [j] [60]; [k] [59]

Relative to the results obtained by Saper et al. [9], the subset of Ayurvedic THPs in our study included more THPs (46 (19%) of 242) with estimated mercury intakes above the TWI, which also exceeded the number of THPs with estimated arsenic or lead intakes above the TWI (22 (9%) and 10 (4%), respectively) (Table 8). A similar distribution of mercury, arsenic and lead among Ayurvedic THPs is found in the official Ayurvedic formulary, which includes more formulas with mercury (23%) than arsenic (7,3%) or lead (2,5%) [34, 35]. In 2008, Saper et al. [65] published a study on mercury, arsenic and lead in Ayurvedic THPs purchased via the Internet in 2005. Lead, mercury and arsenic were found in 40 (20.7%) of 193 Ayurvedic THPs manufactured in the US and India. The intake estimates for mercury, arsenic and lead for all THPs that contained these elements, exceeded selected standards [65]. McElvaine et al. [66] studied metal and metalloid contents of 22 East Indian THPs sampled in India. Arsenic was found in 9 (41%) of the THPs, lead and mercury were each detected in 14 (64%) of the samples and high cadmium levels were shown in 2 products. Maximum and median arsenic and mercury concentrations, considerably exceeded values found in our study but intake estimates were not provided. Garvey et al. [67] sampled 54 Chinese and Southeast Asian THPs in Vietnam, Hong Kong and the US and found in 36 (67%) THPs levels of arsenic, lead and mercury that would result in intake estimates above safety limits that were similar to limits selected for our study. In a study from 2007 by Cooper et al. [43], at least 160 (65%) of 247

Chinese THPs sampled on the Australian market were contaminated with mercury, lead or arsenic. Background exposure to mercury, arsenic or lead from other foods was subtracted from the TDI, thereby reducing room for mercury, arsenic or lead intake from THPs, and a conservative JECFA PTWI for total mercury [68] was applied. In total 3% of the Chinese THPs contained mercury, arsenic or lead levels resulting in intakes at the recommended dose that would exceed the selected TDI estimates by 20-fold to up to 2750-fold [43]. In our study 5 (12%) of 42 Chinese THPs could result in estimated mercury, arsenic or lead intakes exceeding safety limits in a range of 1.1-fold to more than 2000-fold (Tables 7 and 8).

In order to identify THPs possibly high in mercury, arsenic or lead beforehand, we screened the labeling for traditional names for Asian THPs or traditional names for ingredients as listed in Tables 1-3, which can contain arsenic, lead or mercury. Of the 59 THPs in our study that would result in mercury, arsenic or lead intakes significantly above TWI limits, the labeling of 25 (42%) carried indications from Tables 1-3, which could suggest high levels of these elements (Table 7). Our sampling protocol, which includes Tables 1-3, was therefore helpful in identifying products with significant mercury, arsenic or lead levels, which carried traditional product names or ingredients on the labeling. However, it proved not to be effective in identifying high-risk THPs with incomplete ingredient lists or modern product names. Our data suggests that consumers cannot rely on the labeling for avoiding Asian THPs with high amounts of mercury, arsenic or lead and that there is a significant risk of inadvertent exposure to these elements from Asian THPs.

In order to estimate the risks associated with exposure to mercury, arsenic or lead from Asian THPs appropriate safety limits have to be selected. Table 9 shows that the selection of a safety limit considerably influences the outcome of the study, which should be considered when interpreting results of studies into mercury, arsenic or lead in THPs. It is pertinent that the differences in toxicity of the various species of an element are taken into account [39]. Reports on the speciation of mercury, arsenic or lead in Asian THPs are scarce. Establishing the speciation of elements is less straightforward for Ayurvedic THPs then for Chinese THPs because Ayurvedic THPs are subjected to more rigorous processing steps that include heat treatments. The ultimate mercury, arsenic or lead species in Ayurvedic THPs are therefore more likely to differ from the initial species in the starting materials.

Mercury from Ayurvedic THPs requires special consideration. Purified mercury (parada) is used as the starting material for most mercury containing Ayurvedic THPs. Elemental mercury shows a low oral toxicity when compared to organic and inorganic mercury compounds [57, 69]. To test if Ayurvedic THPs contained any residual elemental mercury we performed a study on 23 Ayurvedic THPs in which we had shown mercury to be present. No elemental mercury was detected in these products (the results of this study are presented and discussed in Chapter 6 of this thesis), which indicates that all mercury detected relates to either inorganic or organic mercury. For the preparation of Ayurvedic THPs, mercury is mixed with sulfur until kajjali (black mercuric sulfide, HgS) is formed [34-36, 41]. Mercury reacts with sulfur, which forms HgS [70, 71]. The presence of HgS in Ayurvedic THPs was analytically confirmed for the important Ayurvedic mercury preparation Makaradhwaja [38], which is based on kajjali [36]. The preparation was shown to contain 85.3% mercury and 14.2% sulfur matching the stoichiometry of HgS [38]. Use of mercuric sulfide in an Indian ethnic remedy in a daily dose of 180-252 mg

during 4 weeks resulted in adverse effects consistent with a mercury poisoning [17]. Inorganic mercury poisonings from oral use of Chinese THPs with calomel (mercurous chloride, Hg_2Cl_2) [16] and cinnabar (used long-term) have been recorded [72]. Studies on rats and guinea pigs have shown that mercuric sulfide and its natural form cinnabar are bioavailable and neurotoxic [32, 73, 74]. Because methylmercury is primarily formed through methylation by microorganisms [57, 75] and because methylmercury is not used in traditional medicines [72] we considered the presence of this compound in toxicological relevant quantities to be unlikely. The JECFA PTWI for total mercury used by Cooper et al. [43] is mainly applicable to diets with high consumption of fish with high levels in methylmercury [56] and does not necessarily apply to foods high in inorganic mercury [68]. Therefore, a TDI for inorganic mercury [57, 58] was selected for the risk assessment of mercury from Asian THPs. Although this TDI is based on a NOAEL for inorganic mercuric chloride it also applies to other inorganic mercury compounds such as mercuric sulfide [57, 58].

Arsenic is used in Chinese THPs in inorganic form [10, 40, 42, 43]. In the Ayurvedic Formulary, 7% of the formulas contain realgar (manahsila, As_4S_4) and orpiment (talaka, As_2S_3) [34, 35]. From talaka a bhasma is made. Talaka bhasma was reported to contain As_2S_3 [38]. Furthermore, arsenic trioxide (As_2O_3) was found in two kushtas from India and Pakistan [45]. Arsenic trioxide is significantly more toxic than orpiment and realgar [44]. Inorganic arsenic compounds are more toxic than several organic arsenic compounds, notably those compounds (e.g. arsenobetaine) which can be found in seafood in considerable quantities and which are not of major toxicological concern [76]. Cases of arsenic poisoning involving Indian preparations have been reported in Italy [77] and in the UK [17]. The case in the UK involved arsenic intakes with the Indian preparations of up to 210 mg As_2O_3 per day for more than 4 months, causing symptoms consistent with inorganic arsenic poisoning [17]. An Indian case of arsenic poisoning showed keratosis after 6 months of use of several Ayurvedic preparations with significant arsenic levels and after 18 months of use non-cirrhotic portal hypertension was diagnosed [31]. Several chronic arsenic poisonings with Chinese THPs have been reported, which resulted in cutaneous manifestations such as malignancies and kerotic plaques [29, 78-80]. We selected a TDI for inorganic arsenic set by RIVM for the risk assessment of arsenic from Asian THPs. The TDI is based on the JECFA PTWI for inorganic arsenic [60] to which an extra uncertainty factor of 2 was applied for observation errors in epidemiological studies [58].

Lead in Ayurvedic and Chinese THPs is mostly found in inorganic form. Reported forms are PbS and PbO, which can cause intoxications [37, 52, 53, 81]. A large number of lead poisonings with Ayurvedic THPs has been reported [15, 17, 19, 20, 24, 27, 28, 30, 46, 82-84]. These reports contradict the claim that lead in Ayurvedic THPs is detoxified. A case of lead poisoning with a Chinese THP has been recorded in Hong Kong [25]. The US Environmental Protection Agency (US EPA) did not establish an oral reference dose (RfD) for lead nor was the Agency for Toxic Substances and Disease Registry (ATSDR) able to derive a minimal risk level (MRL) for lead, because both agencies could not define a clear threshold for some of the health effects linked to exposure to lead [85, 86]. RIVM set a TDI for lead and lead compounds [58], which is directly derived from the JECFA PTWI for lead from all sources [54]. We selected therefore the PTWI set by JECFA [54] for the risk assessment of lead from Asian THPs.

Our study shows that the mercury, arsenic and lead contents of Asian THPs are still a cause for concern and that Asian THPs should routinely be tested for metals and metalloids. The VWA will continue to monitor metals and metalloids in Asian THPs on the Dutch market and enforce the safety of these products.

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CHAPTER
6

Based on:

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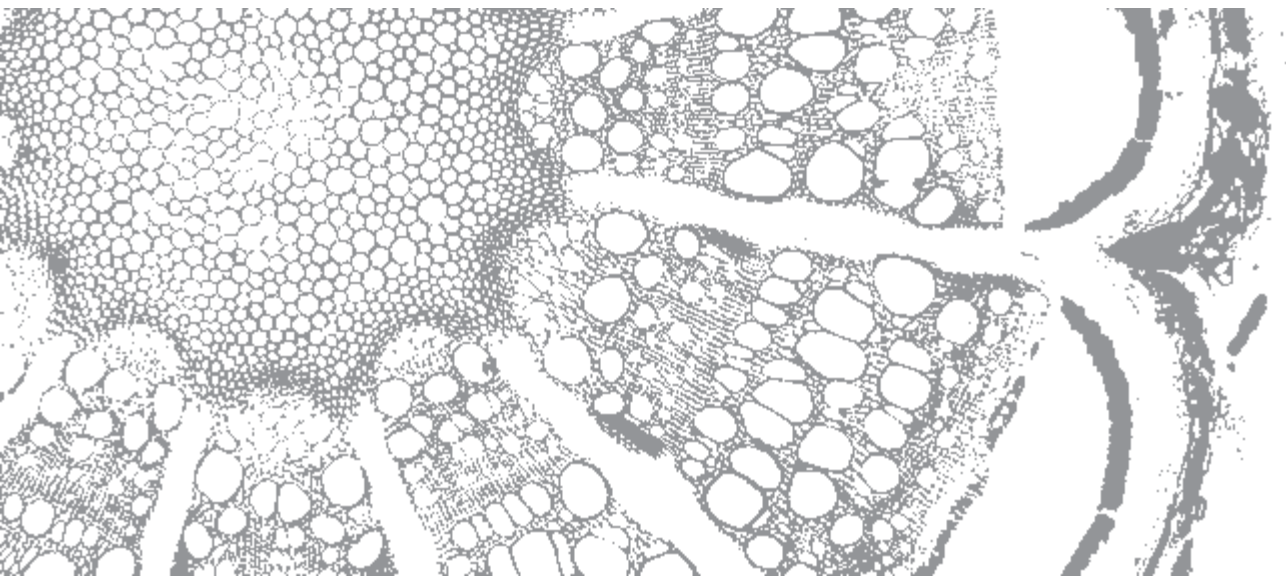
Detection of elemental mercury in selected traditional Ayurvedic herbal preparations

- Article in preparation -

Detection of elemental mercury in selected traditional Ayurvedic herbal preparations



Abstract

Traditional herbal preparations (THPs) play an important role in Ayurveda and other Indian traditional medicine systems. Ingredients high in metals or metalloids are intentionally added to several Ayurvedic and other Indian THPs. The Dutch Food and Consumer Product Safety Authority (VWA) recently reported that 19% of 242 Ayurvedic THPs analyzed between 2004 and 2008 would result in mercury intakes above a toxicological safety limit for inorganic mercury when used at the recommended dose level (Chapter 5). The present study is aimed at investigating whether (part of) the mercury detected in these Ayurvedic THPs is present as elemental mercury (Hg(0)), which shows a low oral toxicity, providing an argument to reconsider the safety assessment using limits for inorganic mercury. To test this, we developed a method that enabled detection of Hg(0) in these Ayurvedic THPs and subsequently performed a study on the presence of Hg(0) in 19 Ayurvedic THPs of which our previous study showed that mercury intakes with the recommended daily dose exceeded the safety limit for inorganic mercury. The results obtained reveal that in 11 of the 19 THPs analyzed all mercury detected could be accounted for by other forms than Hg(0). Extraction of the other 8 THPs with 7M HNO_3, a method demonstrated to efficiently extract Hg(0), revealed that most of the mercury present in these samples (i.e. 69-99%) could not be extracted by 7M HNO_3, pointing at other forms than Hg(0) being present. It is concluded that in all THPs mercury is not mainly present in its relatively non-toxic elemental form, that the mercury detected in Ayurvedic THPs is likely to be present in the inorganic form and that therefore risk assessment based on the safety limits for inorganic mercury are justified.

Introduction

The main Indian traditional medicine systems are Ayurveda, Unani-Tibb and Siddha of which Ayurveda is best known in Western countries [1, 2]. Traditional herbal preparations (THPs) used in these systems can contain significant amounts of mercury, lead and arsenic [1, 3, 4]. We conducted a survey between 2004 and 2008 into the presence of metals and metalloids in Ayurvedic and other Asian THPs (Chapter 5 of this thesis). In the survey presented in Chapter 5, mercury was detected in 117 out of 242 Ayurvedic THPs and use at the recommended dose level of 46 of these would result in total mercury intakes above a toxicological safety limit for inorganic mercury [5]. In Ayurveda, therapeutic qualities are attributed to mercury and this element is added to several Ayurvedic THPs in the form of preparations called "bhasmas" [6-10]. Metals and metalloids in bhasmas and kushtas, comparable preparations used in Unani-Tibb, are claimed to be detoxified by rigorous processing [4, 6, 8-12].

The chemical species in which a metal or metalloid exists can significantly affect its toxicity [13]. The main types of mercury species are elemental mercury (Hg(0)), inorganic and organic mercury compounds. Mercury is present in inorganic mercury compounds as monovalent or divalent mercury (expressed as Hg (I) and Hg (II) or Hg^{1+} and Hg^{2+}). Organic mercury results from the combination of carbon and mercury [14]. Inorganic mercury is nephrotoxic and neurotoxic and long-term use of preparations with inorganic mercury compounds has resulted in poisonings worldwide [5]. Methylmercury (CH_3Hg^+) can be formed by aquatic microorganisms and this highly toxic organic mercury species can diffuse through phospholipid membranes [13, 14]. In contrast, Hg(0) shows a low oral toxicity when compared to organic and inorganic mercury compounds [5, 15, 16]. Therefore, detoxification of mercury should in theory imply conversion of inorganic and organic forms of mercury into Hg(0). The speciation of mercury in Ayurvedic and other Indian THPs is not well known but available reports point mainly to mercuric sulfide (HgS) [12, 17, 18]. A case of human mercury poisoning linked to the use of an Indian remedy with HgS has been reported [17].

Parada or purified mercury (most likely present as Hg(0)) is used in 20% of the 634 formulas for Ayurvedic THPs in the official Ayurvedic Formulary of India, and 4% include hingula (cinnabar - HgS) [9, 10]. When used in THPs, parada is first ground with equal parts of sulfur with mortar and pestle until it is completely transformed into a very fine black powder called kajjali [19]. Kajjali consists of HgS [18]. Makaradhwaja, an important Ayurvedic mercury preparation based on kajjali [19], was shown to contain HgS [12, 20]. It is not well known whether Hg(0) is completely transformed into mercury compounds during the preparation of Ayurvedic THPs. Hg(0) and sulfur can form HgS at low temperatures. In an experiment, both elements were thoroughly mixed in a shaker in an S/Hg molar ratio of 1.5 at room temperature and low humidity. After 37 months of storage under the same conditions, 20% of the mixture consisted of mercuric sulfide [21]. In another experiment [22], equal weights of Hg(0) and sulfur (S/Hg molar ratio of 6) were mixed for several hours under anaerobic conditions at approximately 40 ºC with quartz cobbles to enhance agitation. The mercury completely reacted, yielding black mercuric sulfide. A molar excess of sulfur to Hg(0) facilitates a faster reaction [22]. During mixing of both elements under aerobic conditions, mercury oxide or mercury salts may form, but these compounds may also be already

present in the Hg(0) [22]. Because the preparation of kajjali, which also includes ample agitation and a sixfold molar excess of sulfur to mercury, resembles the production process of HgS in the last experiment, it is likely that kajjali also consists of HgS. But because kajjali is prepared under aerobic conditions other mercury compounds may also be formed.

An important aspect in the risk assessment of mercury from the Ayurvedic THPs was whether mercury in these THPs was present as Hg(0), which shows a low oral toxicity. A relative high Hg(0) level would therefore require another type of safety limits than the safety limits for inorganic mercury [5] previously used in the risk assessment presented in Chapter 5 of this thesis. The objective of the current study was therefore to determine whether mercury found in Ayurvedic THPs was present as the non-toxic Hg(0) or not, in order to facilitate and further support the selection of established toxicological safety limits to be used for risk assessment providing the basis for subsequent market interventions. To this end a method enabling detection of Hg(0) in the Ayurvedic THPs was established followed by detection of the possible presence of Hg(0) in a series of 19 THPs which were previously shown to contain significant levels of mercury in the survey presented in Chapter 5.

Material and methods

Sampling

For this study 19 Ayurvedic THPs that were previously shown to contain significant levels of mercury were selected as well as 2 Ayurvedic THPs that were previously shown to contain relatively low levels of mercury (Chapter 5 of this thesis). The THPs were sampled from 2 suppliers in the Netherlands and produced in Nepal and India. The labeling of most of these products carried the names of classical formulations described in official Ayurvedic handbooks and traditional ingredients [9, 10]. We therefore concluded that these products could serve as models for establishing whether elemental mercury (Hg(0)) was present in these Ayurvedic THPs. Of each THP, at least 20 g of material was sampled.

ICP-MS determination of total mercury (Hg (T)) following microwave-assisted extraction

The analytical method used by the VWA to determine total mercury (Hg(T)) in the 19 Ayurvedic THPs selected for the current study, has been described previously in our report on lead, mercury and arsenic in Asian THPs (Chapter 5 of this thesis). The method can be summarized as follows. In a quartz vessel, 3 ml nitric acid (HNO_3, 65%) and 1 ml hydrogen peroxide (H_2O_2, 30%) were added to 20 mg of powdered sample. In order to test if each destruction vessel was clean prior to use, test runs were performed on each vessel with only 3 ml HNO_3 (65%) and 1 ml H_2O_2 (30%) before each series of samples. This was repeated after each series in order to confirm that the vessels did not contain any residual mercury. The vessels were placed in high-pressure digestion vessels, which were then closed. Next a microwave-assisted extraction was performed in a microwave oven (Ethos Plus Microwave Labstation, Milestone) equipped with temperature and pressure feedback control. In the microwave oven, the samples were brought in 15 minutes from room temperature to 200ºC and were then treated for 25 minutes at that temperature after which the samples were allowed to cool to room temperature. The

translucent solution produced in the microwave-assisted extraction process was then quantitatively transferred with de-ionized water from the extraction vessel to a 50 ml volumetric flask. Before filling the flask to the 50 ml mark with de-ionized water, 500 µl aureumchloride (AuCl, 100 mg/L) solution was added to prevent a build-up of persistent mercury residues in the ICP-MS system (Thermo Electron PQ Excell) used to determine the mercury levels in the samples (Chapter 5 of this thesis). Throughout the analysis Milli-Q grade de-ionized water was used. The sample solutions were diluted (up to 10,000 times) when necessary to fit within the calibration curve. ICP-MS measurement was performed using the 'Collision Cell Technology mode' (CCT-mode) with a helium/hydrogen flow (95/5 %). The accuracy of the method was determined by analysis of a Certified Reference Material (CRM)(IRMM; BCR 482: trace elements in Lichen). For mercury the accuracy of the method is about 100% and the RSD_r was found to be 9.5%. The LOD of mercury was 0.03 mg/kg, the LOQ was 0.06 mg/kg and the expanded measurement uncertainty was 0.047 mg/kg.

Extraction with 6M HCl and 7M HNO_3 to determine the absence or presence of Hg(0) in THPs

After the determination of the Hg(T) content of the THPs using ICP-MS as described above, it was investigated whether a significant part of the Hg(T) detected would be present as Hg(0). To this end a method was developed in which i) 7M HNO_3 was shown to extract mercury present in Ayurvedic THPs including Hg(0) and in which ii) 6M hydrochloric acid (HCl) was shown to extract mercury present in Ayurvedic THPs excluding Hg(0). The two extraction methods together will then provide insight in the possible presence of Hg(0) in Ayurvedic THP samples.

To test the specificity of the method for determining the absence or presence of Hg(0) in Ayurvedic THPs, Hg(0) or mercuric(II)chloride ($HgCl_2$) were added to two different Ayurvedic THP samples with relatively low endogenous mercury levels (Ayu-Digeste and Ayu-Geripower) after which the recovery of these mercury species with the two extraction methods was determined. In general, the extraction procedures consisted of extracting 0.5 g of test sample with either 25 ml 6M HCl or 25 ml 7M HNO_3 both at 100 °C for 60 minutes. After extraction the extract solution was quantitatively transferred with de-ionized water (Milli-Q) to a 50 ml volumetric flask.

The sample preparation was as follows. From one THP sample (Ayu-Digeste), two groups of test samples were prepared in triplicate. To the first group of test samples from this THP Hg(0) was added (60-130 g/kg) and then each sample was split. One part was extracted with 6M HCl and the other part with 7M HNO_3 as described above. $HgCl_2$ was added to the second group of test samples (13-40 g/kg). Each of the test samples to which $HgCl_2$ was added, was then split in two and one part was extracted with 6M HCl and a second part with 7M HNO_3. The addition of Hg(0) was repeated in test samples prepared from a second THP sample (Ayu-Geripower). To this test sample 30 g/kg Hg(0) was added and the test sample was split in two. One part was extracted with 6M HCl and the other part with 7M HNO_3 as described above. The mercury contents of the various extractions were subsequently analyzed using ICP-MS.

When the specificity of the method to detect the absence or presence of Hg(0) in THPs was demonstrated the 19 THPs shown before to contain significant levels of mercury were analyzed for the absence

or presence of Hg(0). To this end, a part (0.5 g) of the sample was extracted with 6M HCl and a second part (0.5 g) with 7M HNO_3. Subsequently, the mercury contents of both extraction solutions were analyzed by ICP-MS.
Each sample was extracted twice with each of the three extraction methods, i.e. the microwave assisted extraction method for Hg(T), and the 6M HCl and 7M HNO_3 based extraction procedures. The reported values for each extraction method are the mean of two extractions. The standard deviation of paired measurements was calculated using the equation $s = \sqrt{[(\Sigma d^2)/ 2k]}$ where 'd' is the difference of duplicate measurements and 'k' is the number of sets of duplicate measurements. The relative standard deviation (RSD) was calculated for each extraction method by dividing the standard deviation of paired measurements by the mean of all results obtained with the extraction method.

Results

Specifity of the method to determine the absence or presence of Hg(0) in THPs

The specificity of the acid extraction method to detect either the presence or absence of Hg(0) was tested by addition of Hg(0) to two THP samples with relatively low mercury contents (Ayu-Digeste and Ayu-Geripower) containing respectively 0.04 and 0.01 g/kg of mercury when analyzed by the method for determination of Hg(T). From each of these samples, test samples were prepared and Hg(0) was added to these two groups of test samples and the test samples thus obtained were split in two and were extracted with either 6M HCl or 7M HNO_3 as described in the methods section. Mercury levels in these extracts were then measured by ICP-MS.

Table 1 Mercury levels detected in extracts from THP samples spiked with $HgCl_2$ or Hg(0) and extracted with either 6M HCl or 7M HNO_3 ; values presented are the mean +/- SD of N = 2 or 3 independent analyses

			HCl 6M [a]				HNO_3 7M [b]			
THP	Addition	N	Hg (g/kg)	SD	Hg extractability (%)	SD	Hg (g/kg)	SD	Hg extractability (%)	SD
Ayu-Digeste	-	3	0.040	0.001			0.043	0.001		
	Hg(0)	3			0	0			99	3.4
	$HgCl_2$	3			99	1.3			97	1.4
Ayu-Geripower	-	2	0.003	0.002			0.001	0.002		
	Hg(0)	2			0.1	0.2			97	1.5

[a] Extraction of 0.5 g sample with 25 ml of 6M HCl at 100 °C for 60 minutes

[b] Extraction of 0.5 g sample with 25 ml of 7M HNO_3 at 100 °C for 60 minutes

Table 1 presents the recovery (%) of Hg(0) added to these test samples prepared from Ayu-Digeste and Ayu-Geripower. In the first sample (Ayu-Digeste) Hg(0) was almost absent (0-0.03%) in the 6M HCl-extract, but fully extracted by 7M HNO_3 (96-103%). For the second sample (Ayu-Geripower) similar results were obtained. In this sample Hg(0) was also almost absent (0-0.3%) in the 6M HCl-extract but fully extracted by 7M HNO_3 (96-98%). Subsequently, $HgCl_2$ was added to Ayu-Digeste. This mixture was then extracted with 6M HCl and 7M

HNO_3 as described in the methods section, followed by determination of the mercury levels of the extracts by ICP-MS. This was repeated twice. Table 1 presents the results obtained, and reveals that the recovery of $HgCl_2$ upon 6M HCl and 7M HNO_3 extraction amounted to almost 100% for both extraction solvents.

Together these data illustrate that 6M HCl could not extract Hg(0) whereas 7M HNO_3 effectively extracts Hg(0) from THPs. This confirmed that the methods can be used to detect the presence of Hg(0), because the absence of Hg(0) can be derived from i) the situation where the 6M HCl extraction results in Hg levels similar to those detected by the method for quantification of Hg(T) and ii) the absence of significant Hg levels in extraction with 7M HNO_3. With respect to the latter situation it is important to stress that although the experiments presented in Table 1 indicate that like Hg(0) also $HgCl_2$ can be efficiently extracted by 7M HNO_3, it has been demonstrated before that extraction by HCl or HNO_3 of HgS, the form of mercury most likely to be present in Ayurvedic THPs, is highly variable and depending on the matrix, extraction conditions and the sample extracted [23].

Determination of the absence or presence of Hg(0) in selected Ayurvedic THPs

Before the 19 commercial Ayurvedic THPs were analyzed for the presence of Hg(0) by acid extraction with 6M HCl and 7M HNO_3, the total mercury (Hg(T)) levels in these samples were determined. Hg(T) was extracted by microwave-assisted extraction with 3:1 (v/v) HNO_3 (65%) and H_2O_2 (30%) for 25 minutes at 200ºC and analyzed by ICP-MS (Table 2). The first column of Table 2 presents the Hg(T) levels of the 19 THPs obtained by the microwave-assisted extraction method. Each value is a mean of two independent extractions of the same sample by different analysts. The RSD of paired Hg(T) measurements was 24%. Next, parts of each sample were separately extracted with 6M HCl and 7M HNO_3, after which the mercury levels of these extracts were determined by ICP-MS. The results obtained by both extraction procedures are presented in Table 2 as well. The values presented in this table for mercury levels obtained by HCl and HNO_3 extractions, represent a mean of two independent extractions. The RSD of paired measurements of 6M HCl extracts was 9% and the RSD of paired measurements of 7M HNO_3 extracts was 16%.

The results of the Hg(T) microwave-assisted HNO_3/H_2O_2 extraction procedure and the extraction with 6M HCL were comparable (Table 2). Paired sample T-test for mercury levels found with the Hg(T) extraction procedure and the HCl extraction procedure showed no significant difference ($P>0.05$). The mean of the mercury contents obtained by the Hg(T) extraction procedure (65 g/kg) was higher than the mean mercury level in the HNO_3 extracts (17 g/kg). Paired sample T-test for mercury levels found with the Hg(T) extraction procedure and the HNO_3 extraction method showed a significant difference in results of both extraction methods ($P<0.05$). The mean mercury contents of HCl extracts (57 g/kg) was higher than the mean mercury level found in the HNO_3 extracts (17 g/kg). Paired sample T-test for mercury levels found in the HCl extracts and the HNO_3 extracts showed a significant difference in results of both extraction methods ($P<0.05$).

Closer comparison of the data obtained by the Hg(T) extraction procedure with the mercury levels detected upon extraction with 6M HCl, a method shown unable to extract Hg(0) from THPs, reveals that for 11 of the 19 samples the mercury levels detected upon 6M HCl extraction are equal to, or even higher than the levels detected upon determination of Hg(T) (sample No. 1 up to and including No. 11 of Table 2). This reflects that in

these 11 samples all mercury present and detected by the Hg(T) method can be accounted for by mercury forms other than Hg(0) thus supporting the absence of Hg(0) in these 11 samples.

Table 2 Mercury levels in different extraction solutions obtained by treating Ayurvedic THPs with different extraction techniques

Ayurvedic THP	Mercury level (g/kg) [a]		
	Microwave-assisted Hg(T) extraction with 3:1 HNO_3:H_2O_2 [b]	Hg in 6M HCl extraction solution [c]	Hg in 7M HNO_3 extraction solution [d]
1. Kamachudamani Ras	25	50	7
2. Gandhadi Yog	4	6	3
3. Ghandramsu Ras	45	69	2
4. Agni Vati	7	11	9
5. Makar Wazra	126	159	163
6. Jeevani Vati	29	36	26
7. Mahabatbiddhonsan Ras	39	46	10
8. Shingorabrak	26	29	5
9. Ras Yog	124	131	2
10. Triyog Misran	13	13	7
11. Heartina	47	47	3
12. Arogya Vardhini Vati	26	24	8
13. Yogendra Ras	194	167	25
14. Sworna Yog	26	19	5
15. Vasant Yog	37	25	3
16. Bat Chintamani	150	84	35
17. Amrit Ras	149	83	1
18. Hridaya Chintamani	98	53	4
19. Swashanti Yog	73	31	3

a) Each value in the table is a mean of two extractions.

b) Microwave-assisted Hg(T) extraction of 20 mg sample in 3 ml HNO_3 (65%) and 1 ml H_2O_2 (30%) for 25 minutes at 200ºC. The RSD of paired Hg(T) measurements was 24%.

c) Extraction of 0.5 g sample with 25 ml of 6M HCl at 100 °C for 60 minutes. The RSD of paired measurements of 6M HCl extracts was 9%.

d) Extraction with 0.5 g sample with 25 ml of 7M HNO_3 at 100 °C for 60 minutes. The RSD of paired measurements of 7M HNO_3 extracts was 16%.

For the remaining 8 samples (sample No. 12 up to and including No. 19 of Table 2: Arogya Vardhini Vati, Yogendra Ras, Swoma Yog, Vasant Yog, Bat Chintamani, Amrit Ras, Hridaya Chintamani and Swashanti Yog) the mercury levels detected by the 6M HCl method were 42-92% of their respective Hg(T) levels thus leaving the possibility for the presence of Hg(0). However, for these 8 samples the results of the 7M HNO_3 method

demonstrated the absence of significant levels of Hg(0), because upon extraction with 7M HNO_3, a method shown to effectively extract Hg(0) from THPs, 69-99% of the Hg(T) mercury detected was not extracted. For these remaining 8 THPs the mercury levels detected by the 7M HNO_3 method amounted to only 1-31% of the Hg(T) levels, indicating that also in these samples the mercury is mainly present in other forms than Hg(0).

Discussion

To test if Ayurvedic THPs contain elemental mercury (Hg(0)), we performed a study on 19 Ayurvedic THPs in which we previously found mercury by ICP-MS combined with a microwave-assisted extraction step (Chapter 5 of this thesis). The speciation of mercury in these THPs is pertinent for risk assessment because in contrast to inorganic or organic mercury species, Hg(0) via the oral route shows a low toxicity. In the current study the presence of elemental mercury was determined by performing additional acid extractions of mercury with 6M HCl and 7M HNO_3. The mercury content of the extracts was subsequently analyzed with ICP-MS. It was shown that 6M HCl does not extract Hg(0) whereas 7M HNO_3 effectively extracts Hg(0) from THPs. This demonstrated that the methods, combined with quantification of Hg(T) upon microwave assisted HNO_3/H_2O_2 extraction, could be used to establish the absence or presence of Hg(0). Extraction of 19 Ayurvedic THPs previously shown to contain significant levels of mercury, with 6M HCl and 7M HNO_3 showed that for 11 of the 19 samples the mercury levels detected by the 6M HCl extraction method were equal to or even higher than the total mercury contents (Hg(T)) quantified by microwave-assisted extraction. This indicates that these samples did not contain significant levels of Hg(0). For the remaining 8 THPs the mercury levels detected by the 7M HNO_3 method did not reveal high levels of Hg(0) since the amount of mercury detected amounted to only 1-31% of the Hg(T), indicating that also in these samples the mercury is mainly present in other forms than Hg(0).

The fact that for most of the 19 samples the overall level of mercury detected upon extraction with 7M HNO_3 was low compared to the Hg(T) levels could be explained by i) the absence of Hg(0) as the major mercury species present and ii) the preferential presence of for example HgS, the form of mercury most likely to be present in Ayurvedic THPs and reported to be soluble in HCl and HNO_3 to a varying extent depending on the matrix characteristics and extraction conditions [23-25]. However, the variation found between the individual measurements of each sample was relatively high for each of the three extraction methods. Due to the very high mercury levels found in the samples (up to 194 g/kg) the test solutions had to be diluted several times to prevent persistent mercury residues in the ICP-MS system. The high dilution factors required to prevent build-up of these residues were a possible source of the observed variation. Furthermore, inhomogeneity of the samples also represented a potential source of variation in this study. This was especially the case for the Hg(T) method because the sample weight used for Hg(T) analysis was relatively low (20 mg). The differences between the mercury levels found for each sample with the three extraction methods can therefore also be explained to some degree by the variation between the individual measurements of each sample.

Fernández-Martinez and Rucandio [23] determined the solubility of a number of mercury species both in 50% v/v HCl (37.5%) and in 50% v/v HNO_3 (65%), equivalent to respectively 6M HCl and 7M HNO_3. They

reported that most mercury species, including $HgCl_2$, were quantitatively dissolved in both acids with the exception of HgS. This is in line with our finding that both acids could completely extract $HgCl_2$ from a herbal preparation matrix to which this species was added. Moreover, additional findings of Fernández-Martinez and Rucandio [23] pointing to an influence of matrix characteristics and extraction conditions on HgS solubility would support the assumption that a high proportion of Hg(T) in our samples is HgS which in turn would explain the low and variable extraction efficiencies with 7M HNO_3 relative to 6M HCl obtained in our study. Literature information on the speciation of mercury in Ayurvedic THPs also points to the presence of mainly HgS in Ayurvedic preparations with high Hg(T) levels (as discussed in the introduction and in Chapter 5 of this thesis). While neither pure HgS nor its mineral form cinnabar were found to be soluble in 50% v/v HNO_3 (65%) or in 50% v/v HCl (37.5%) by Fernández-Martinez and Rucandio [23] they showed that the solubility of HgS in each solution was significantly higher, but to different degrees, in the presence of certain other compounds and at a relatively high extraction temperature (70 ºC). Relative to the HNO_3 extraction method, the extractability of mercury was promoted by more compounds in the HCl extraction method [23]. In the 50% v/v HCl (37.5%) solution, KI, MNO_2 and $NaNO_3$ drastically promoted the solubility of HgS at room temperature, almost leading to quantitative recoveries. At high temperature (70 ºC) all individually tested reagents (KI, KCl, $FeCl_3$, Fe_2O_3, $CuSO_4$, $FeSO_4$, MNO_2, $NaNO_3$) were able to promote the solubility of HgS in an HCl solution to a significant degree. In 50% v/v HNO_3 (65%), quantitative recoveries of HgS were only obtained in the presence of chloride ions both at room temperature and at high temperature. At room temperature no other of the reagents tested could increase the solubility of HgS in 50% v/v HNO_3 (65%). Raising the extraction temperature to 70 ºC resulted only in partial dissolution of HgS in 50% v/v HNO_3 (65%) in the presence of KI while the rest of the reagents resulted in a low ($FeSO_4$) or no notable increase in the solubility at high temperature. This would suggest that the effect of higher extraction temperatures on the extraction efficiency with 50% v/v HNO_3 (65%) is modest. Furthermore the Fernández-Martinez and Rucandio study [23] explored the effect of the matrix on the extractability of HgS by testing the extractability of HgS in soil at room temperature. The effect of the matrix on the extractability of HgS was much more pronounced in 50% v/v HCl (37.5%) than in 50% v/v HNO_3 (65%). Only when the soil sample was spiked with halide compounds, the solubility of HgS in 50% v/v HNO_3 (65%) was increased to partial dissolution [23].

It is plausible that in our study the low and variable extraction efficiencies found at 100 ºC with 7M HNO_3 compared to 6M HCl were caused by differences in the composition of the THPs tested. However, Hg levels in HNO_3 extracts of a few samples approached (Agni Vati and Jeevani Vati) or were equal (Makar Wazra) to Hg levels in their respective HCl extracts which could have been caused by relatively high levels of compounds such as halides in the THP matrix that could increase the solubility of Hg in HNO_3 if this Hg would be mainly present as HgS as suggested by literature. The other samples in our study showed low ratios of Hg in HNO_3 to Hg in HCl extracts which were equal to or lower than 50% in 16 THPs, suggesting that these THPs contained lower levels of compounds that could increase the solubility of HgS in 7M HNO_3.

Iron, chloride and manganese were reported to be present in significant amounts in several bhasmas in a study on the chemical composition of these Ayurvedic metallic-herbal preparations used in many Ayurvedic

THPs [12]. Fernández-Martinez and Rucandio [23] showed that iron, chloride and manganese compounds could promote the solubility of HgS in HCl and HNO_3, but each to a different degree dependent on the extraction solution and extraction conditions. The levels of these compounds could vary considerably between different types of bhasmas. The iron, chloride and manganese levels in bhasmas ranged from not detected up to 4 g/kg for chloride, 11 mg/kg for manganese and 570 g/kg for iron in an iron-based bhasma [12]. Finally, it is also important to note that organic mercury, including for example methylmercury (CH_3Hg^+), is not reported to be used as a mercury source in traditional medicine and is mainly formed by aquatic microorganisms through methylation, under conditions not likely to occur during preparation of traditional medicines (discussed in Chapter 5 of this thesis).

In summary, the high extractability of Hg from Ayurvedic THPs in 6M HCl found in our study would be in line with the results obtained by Fernández-Martinez and Rucandio [23] when the Hg in Ayurvedic THPs is mainly present as HgS. These authors showed that at high extraction temperatures a broader range of compounds could increase the solubility of HgS to a higher degree in 6M HCl than in 7M HNO_3, which could explain that in our study the mercury levels in the HCl extracts were comparable to the Hg(T) levels obtained by the microwave assisted extraction method. Altogether, it is concluded that in all THPs mercury is not mainly present in its relatively non-toxic elemental form Hg(0) and that the mercury detected in Ayurvedic THPs is most likely present in an inorganic form and that therefore risk assessment based on the safety limits for inorganic mercury are justified.

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CHAPTER
7

Based on:

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Monitoring of Polycyclic Aromatic Hydrocarbons (PAH) in food supplements with botanicals and other ingredients on the Dutch market

- Article submitted for publication -

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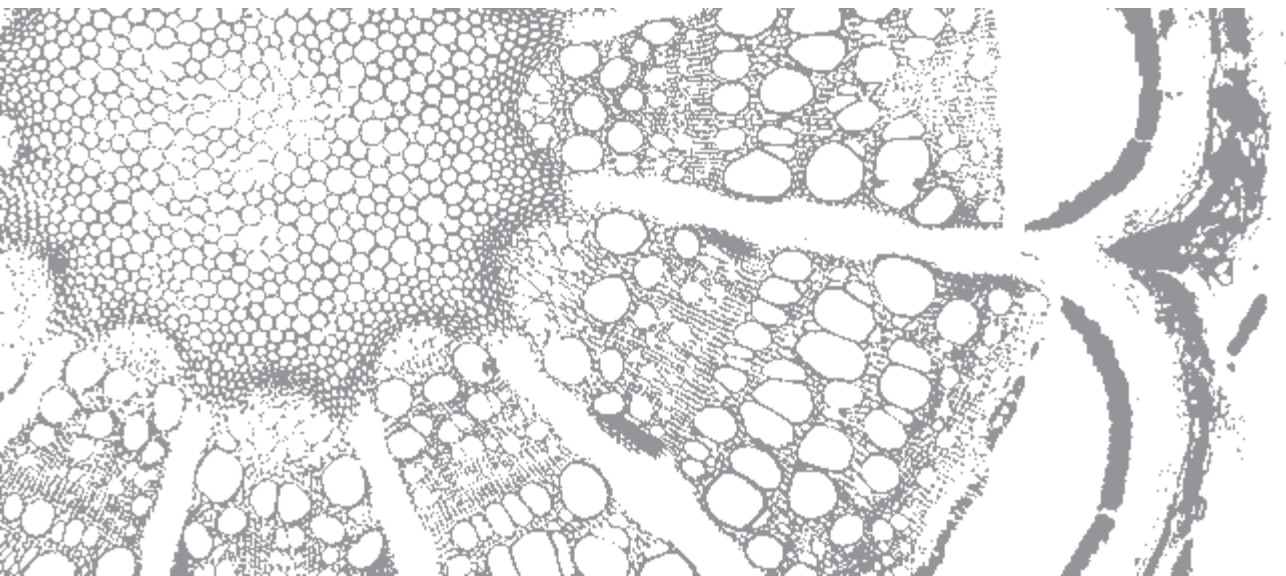

Abstract

Food supplements can contain polycyclic aromatic hydrocarbons (PAH). The European Food Safety Authority (EFSA) has defined 16 priority PAH that are both genotoxic and carcinogenic and identified 8 priority PAH (PAH8) or 4 of these (PAH4) as good indicators of the toxicity and occurrence of PAH in food. The current study aims to determine benzo[a]pyrene and other EFSA priority PAH in different categories of food supplements containing botanicals and other ingredients. In 2003 up to 2008, benzo[a]pyrene exceeded the limit of quantification (LOQ) in 553 (44%) of 1258 supplements with a lower bound mean of 3.37 µg/kg. In 2008 and 2009, benzo[a]pyrene and 12 other EFSA priority PAH were determined in 333 food supplements. Benzo[a]pyrene exceeded the LOQ in 210 (63%) food supplements with a lower bound mean of 5.26 µg/kg. Lower bound mean levels for PAH4 and PAH8 (-indeno[1,2,3-cd]pyrene) were 33.5 µg/kg and 40.5 µg/kg, respectively. Supplements containing resveratrol, *Ginkgo biloba*, St. John's wort and propolis showed relatively high PAH levels in 2008 and 2009. Before 2008, supplements with these ingredients and also dong quai, green tea or valerian contained relatively high benzo[a]pyrene levels. On average, PAH intake resulting from food supplement use will be at the lower end of the range of contributions of main food groups to PAH exposure, although individual food supplements can contribute significantly to PAH exposure. Regular control of PAH levels in food supplements may prove a way forward to further reduce the intake of PAH from food.

Introduction

Polycyclic aromatic hydrocarbons (PAH) can be formed during incomplete combustion or pyrolysis of organic matter and during industrial processes [1-3]. PAH found in food are either derived from the environment or are directly formed during industrial food processing or domestic food preparation [2]. Several PAH such as benzo[a]pyrene are both genotoxic and carcinogenic. Lists of priority PAH were put forward on the basis of their individual genotoxic and carcinogenic properties by the US Environmental Protection Agency (EPA), the Scientific Committee on Food (SCF) of the European Union (EU) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [1, 2, 4, 5]. The SCF identified a priority group of 15 PAH in the risk assessment of PAH in food and concluded that benzo[a]pyrene could be used as a marker for the occurrence and effect of carcinogenic PAH in food [1]. JECFA focused on 13 of these PAH, which were identified to be genotoxic and carcinogenic [2]. Additionally, JECFA noted that besides these 13 PAH, benzo[c]fluorene was also genotoxic and carcinogenic but the Committee did not include this substance in their evaluation because of a lack of data on its occurrence in food [2].

Table 1 Overview of lists of priority PAH put forward by SCF, JECFA and EFSA and the priority PAH proposed by EFSA as indicators for the occurrence and toxicity of PAH in food

PAH			Priority PAH			EFSA indicator groups [c]		
			SCF [a]	JECFA [b]	EFSA [c]	PAH2	PAH4	PAH8
1.	Benz[a]anthracene	BaA	x	x	x		x	x
2.	Benzo[b]fluoranthene	BbF	x	x	x		x	x
3.	Benzo[j]fluoranthene	BjF	x	x	x			
4.	Benzo[k]fluoranthene	BkF	x	x	x			x
5.	Benzo[ghi]perylene	BgP	x	-	x			x
6.	Benzo[a]pyrene	BaP	x	x	x	x	x	x
7.	Chrysene	CHR	x	x	x	x	x	x
8.	Cyclopenta[cd]pyrene	CPP	x	-	x			
9.	Dibenz[a,h]anthracene	DhA	x	x	x			x
10.	Dibenzo[a,e]pyrene	DeP	x	x	x			
11.	Dibenzo[a,h]pyrene	DhP	x	x	x			
12.	Dibenzo[a,i]pyrene	DiP	x	x	x			
13.	Dibenzo[a,l]pyrene	DlP	x	x	x			
14.	Indeno[1,2,3-cd]pyrene	IcP	x	x	x			x
15.	5-methylchrysene	5MC	x	x	x			
16.	Benzo[c]fluorene	BcL	-	+ 1	x			

References: [a] [1]; [b] [2]; [c] [3]

The European Commission requested Member States in Recommendation 2005/108/EC to monitor in certain foods the occurrence of the 15 priority PAH identified by the SCF. These surveys were to cover foods

such as oils and fats, infant formulae and baby foods, meat and marine products for which maximum levels of benzo[a]pyrene are specified in Regulation (EC) No. 1881/2006 and other foods that can contain high levels of PAH, such as dried fruits and food supplements for which no limits yet exist in this Regulation [6, 7]. The data obtained would be used for future review of the suitability of maintaining benzo[a]pyrene as a marker [6]. In 2008 the European Food Safety Authority (EFSA) [3], which continued the work of the SCF, reviewed the available data on occurrence and toxicity of 16 priority PAH, 15 of which were identified by SCF and one, benzo[c]fluorene, by JECFA. Table 1 shows the priority PAH identified by SCF, JECFA and EFSA [1-3]. EFSA concluded that 8 of these 16 'EFSA priority PAH' for which oral carcinogenicity data are available (PAH8) could be used as indicator - either individually or in combination - of the carcinogenic potency of PAH in food (Table 1). However, EFSA established that benzo[a]pyrene alone was not a suitable indicator of the occurrence of PAH in food. EFSA concluded that in its place the most suitable indicators of PAH in food with regards to both occurrence and toxicity were currently PAH4 or PAH8 (Table 1), with PAH8 not providing much added value compared to PAH4 [3].

The Dutch Commodities Act includes since January 2006 a maximum level of 10 µg/kg benzo[a]pyrene for food supplements with botanical ingredients and a maximum of 2 µg/kg benzo[a]pyrene for supplements without these ingredients [8]. These limits were introduced as a result of an ongoing survey by the Voedsel en Waren Autoriteit (VWA - the Dutch Food and Consumer Product Safety Authority) into the presence of benzo[a]pyrene in food supplements [8]. In 2006 we published the analytical method for quantification of benzo[a]pyrene and also benzo[a]pyrene levels in a series of food supplements, mainly supplements containing botanical and fat soluble vitamin ingredients, sampled since 2003 (n=950), and of other food samples (n=400) [5]. More than 30% of the food supplements contained benzo[a]pyrene above 1.2 µg/kg up to 135 µg/kg. Nearly half of 60 samples of raw materials for food supplements (mostly d-alpha tocopherol - natural vitamin E), contained benzo[a]pyrene above 1.2 µg/kg up to 275 µg/kg. Differences in PAH levels between categories of food supplements were not explored in this publication. The primary data suggested however that supplements with natural vitamin E and certain botanical ingredients frequently contained relatively high benzo[a]pyrene levels. In 2005, the UK Food Standards Agency (FSA) determined the 15 SCF priority PAH in 200 food supplements [9]. EFSA published data on the 16 EFSA priority PAH in nearly 300 food supplements that also included the FSA data [3, 4, 9, 10]. In these and in other studies relatively high PAH levels were found in various individual food supplements containing botanical ingredients or bee products such as propolis [4, 11, 12]. However, differences in PAH levels between categories of food supplements were not analyzed further in these reports either.

More detailed data on the variation in PAH levels between supplement categories would aid the setting of European limits for PAH in food supplements. Moreover, this information would also help to focus surveys on PAH in food supplements at those products with potentially high PAH levels. In this respect especially botanical food supplements represent a category of interest. Therefore, the objective of the present study was to collect data on the occurrence of PAH in different categories of food supplements, with special emphasis on botanical food supplements. Furthermore, the analytical method was expanded to include more of the 16 EFSA priority PAH in addition to benzo[a]pyrene.

Material and Methods

Sampling

For this survey food supplements were sampled on the Dutch market by inspectors of the VWA. From 2003 up to and including 2007, 1258 samples of food supplements were collected for benzo[a]pyrene analysis. In 2008 and 2009, 333 food supplements were collected for the determination of benzo[a]pyrene and other EFSA priority PAH. The supplements were collected using a standardized sampling protocol twice yearly. In order to identify groups of food supplements with relatively high PAH levels, the sampling protocol was adjusted each time to include more of those types of supplements that previously showed high PAH levels. Of each sample the name of the manufacturer or distributor, the product name, the date of minimum durability, and the production lot code were routinely entered in the VWA inspection database.

The supplements sampled for this study contained vitamins, botanical ingredients including fatty acids or oils, phytochemicals extracted from plants, fatty acids or oils from animal origin, and other ingredients derived from animals such as glucosamine, chitosan, green lipped mussel extract, and others. EFSA defines the term 'botanical' as all botanical materials such as whole, fragmented or cut plants, plant parts, algae, fungi, and lichens. The term 'botanical preparations' is used by EFSA to refer to all preparations obtained from botanicals by various processes (e.g. pressing, squeezing, extraction, fractionation, distillation, concentration, drying up, and fermentation)[13]. Unless otherwise specified, the term 'botanical' can refer in the current study both to a botanical (or botanical material) or a botanical preparation as defined by EFSA. In 2003 and 2004 the majority of the sampled food supplements were various types of supplements with d-alpha tocopherol (natural vitamin E) and botanical supplements. In the ensuing years (2005-2009) the sampling protocols targeted mainly botanical supplements.

Grouping of supplements

In order to identify whether certain ingredients of food supplements were contaminated with PAH to a higher extent and more frequently than others, sampling was preferentially aimed at supplements with only one main or 'active' ingredient. The 'active' ingredient refers to the ingredient that is clearly regarded by the manufacturer or distributor to show nutritional or physiological activity. The food supplements that were sampled belonged to three main categories: i) essential fatty acids, ii) botanicals, and iii) other substances. Within each main category, subcategories were created on the basis of the active ingredients. Supplements from a subcategory identified by an ingredient name such as *Ginkgo biloba*, contained in principle no other active ingredients. In cases where supplements could be grouped in more than one category these products were placed in the category that best defined the nature of these products. Plant oils could for instance be listed as botanical preparations but were categorized instead as 'essential fatty acids'.

Most types of mono-ingredient supplements of which less than 5 products were sampled, were placed in several general 'mono-ingredient' subcategories. However, some specific subcategories consisting of less than 5 samples were created for mono-ingredient supplements that were of interest due to relatively high or

unanticipated low PAH levels. In only a few cases, botanical supplements with two active ingredients were included in the group of mono-ingredient botanical supplements, but only when the botanical ingredient was accompanied by one other active ingredient (vitamin C, for example) that was not found to contain significant PAH levels in this study. In case PAH were found in those supplements, it was concluded that these PAH were derived from the botanical ingredient.

Food additives, substances added for technical purposes such as bulking and glazing agents, or other 'inactive' ingredients that do not characterize the supplement, were not used to categorize the supplements. Ingredients of food supplements collected in 2008 and 2009 were recorded from the containers of food supplements. Containers of food supplements collected before 2008 were not available, and ingredients of these supplements were identified by searching the Internet by using the product name and the name of the manufacturer of distributor.

Experimental

The analytical method used to determine benzo[a]pyrene in supplements sampled from 2003 up to and including 2007 has been described previously by us [5]. This method was also able to quantify some other EPA PAH. It was based on an online donor acceptor complex chromatography (DACC) cleanup prior to reversed-phase high performance liquid chromatography with fluorescence detection [14]. In 2008 the method was altered to meet the requirements for the analysis of the 16 EFSA priority PAH. This expanded method can be described as follows. The sample was homogenized and 1 g of sample was then transferred to a 20 ml volumetric flask and brought up to mark with 2-propanol. After shaking for 20 minutes at 120 strokes/minute in order to extract the PAH, the extract was filtered by means of a 0.45 µm syringe filter and injected on a DACC column (Chromspher Pi, 80 x 0,3 mm) using 2-propanol as mobile phase. After removal of matrix components using 2-propanol as the eluent, the PAH were stripped from the DACC column by means of ethylacetate – acetonitril (70/30 v/v) and subjected to 2 reversed phase analytical columns in series (Lichrocart 250-4 Lichrospher PAH RP-18 5 µm) prior to quantification of the PAH by variable wavelength fluorescence detection.

Cyclopenta[cd]pyrene and benzo[j]fluoranthene were not included in the method because of low fluorescence intensities. Moreover, indeno[1,2,3-cd]pyrene could also not be included. The analysis of indeno[1,2,3-cd]pyrene would require different wavelength settings but these could not be implemented because of insufficient resolution of this PAH and dibenzo[a,e]pyrene. Use of a multi-channel fluorescence detector combined with a photo diode array detector would have allowed for the determination of these remaining three PAH, but our routine method was not equipped with these features at that time. The main alterations to the method described by us in 2006 [5] were in the mobile phase gradient and the fluorescence wavelength settings and these are presented in Tables 2 and 3, respectively.

Table 2 HPLC Quaternary pump gradient for the analysis of 13 PAH

Time (min)	H_2O (%)	Acetonitril (%)	Ethylacetate (%)	Flow (ml/min)
0.00	15	85	0	0.40
15.00	15	85	0	1.00
26.00	15	85	0	1.00
35.00	5	90	5	1.00
45.00	5	80	15	1.00
50.00	5	80	15	1.00
55.00	0	30	70	1.00
70.00	0	30	70	1.00
73.00	15	85	0	1.00
75.00	15	85	0	1.00
80.00	15	85	0	0.40

Table 3 Variable wavelength settings for the analysis of 13 PAH

Time (min)	λ_{exc} (nm)	λ_{em} (nm)	PAH
0 - 32.0	240	355	benzo[c]fluorene
32.0 - 40.0	260	390	benz[a]anthracene
			chrysene
			5-methylchrysene
40.0 - 56.8	290	430	benzo[b]fluoranthene
			benzo[k]fluoranthene
			benzo[a]pyrene
			dibenzo[a,l]pyrene
			dibenz[a,h]anthracene
			benzo[ghi]perylene
56.8 - 62.0	296	405	dibenzo[a,e]pyrene
			benzo[b]chrysene
62.0 - 66.5	292	435	dibenzo[a,i]pyrene
66.5 - 84	300	450	dibenzo[a,h]pyrene

The method of analysis was in-house validated according to ISO 17025 guidelines. The limit of detection (LOD) was determined based on the signal to noise ratio. The LOD was defined as the concentration at which the peak height corresponds with 3 times the bandwidth of the noise. The limit of quantification (LOQ) was defined as 2 times the limit of detection. During the validation study, performance criteria such as precision under repeatability and reproducibility conditions, trueness, recovery and measurement uncertainty were established (Table 4). During the analyses measurement quality was assured by means of random analysis of standard control samples of food supplements, which were monitored in time by Shewhart control charts.

Table 4 Validation data on the method for the determination PAH in botanical materials

PAH	LOD (µg/kg)	LOQ (µg/kg)	Rec. (%)	True. (%)	RSD_r (%)	R_L (µg/kg)	U (%)
Benz[a]anthracene	1.76	3.53	102	100	8	1.4	10
Benzo[b]fluoranthene	0.76	1.53	99	99	2	0.9	7
Benzo[j]fluoranthene	-	-	-	-	-	-	-
Benzo[k]fluoranthene	0.26	0.53	107	100	9	2.5	18
Benzo[ghi]perylene	0.95	1.90	109	104	2	0.7	10
Benzo[a]pyrene	0.26	0.52	111	111	2	0.5	23
Chrysene	0.39	0.79	100	102	6	0.9	8
Cyclopenta[cd]pyrene	-	-	-	-	-	-	-
Dibenz[a,h]anthracene	1.00	2.01	100	96	3	0.5	10
Dibenzo[a,e]pyrene	0.67	1.34	100	103	4	2.1	17
Dibenzo[a,h]pyrene	0.52	1.04	98	121	27	2.9	47
Dibenzo[a,i]pyrene	0.33	0.65	114	102	3	1.2	9
Dibenzo[a,l]pyrene	0.40	0.79	112	113	2	1.2	27
Indeno[1,2,3-cd]pyrene	-	-	-	-	-	-	-
5-methylchrysene	0.35	0.69	96	98	8	1.5	12
Benzo[c]fluorene	0.58	1.16	110	114	2	2.4	32

LOD: Limit of Detection

LOQ: Limit of Quantification

Rec.: Recovery

True.: Trueness

RSD_r: Relative standard deviation calculated from results generated under repeatability conditions

R_L: Reproducibility, within laboratory

U: Expanded uncertainty

Intake estimates and statistics

Intakes of benzo[a]pyrene, PAH2, PAH4 and PAH8 with a supplement when used at the maximum dose level were estimated for several supplements in which relatively high concentrations of benzo[a]pyrene were found. The daily intake of these PAH with a supplement was calculated from the weight of one unit (determined by preferably weighing 10 or more units), the analytically determined PAH level and the maximum recommended daily use level stated on the label. When a supplement consisted of capsules or soft gels, only PAH levels of its contents were reported and where the weight of a capsule or soft gel was needed for calculations the weight of its contents was taken.

In this study all values lower than the limit of detection (LOD) or limit of quantification (LOQ) were replaced by 0 (lower bound) in order to calculate the descriptive statistics. To aid the comparison of our data with data on PAH in food supplements on the European market published in a report of the EFSA Unit of Data Collection and Exposure in 2007 and the revised and updated report published in 2008, the descriptive statistics

for the benzo[a]pyrene data obtained before 2008 included the calculation of the mean, the median, the 90th and the 95th percentiles (P90 and P95, respectively) and the maximum [10]. Percentiles were only calculated when sample numbers exceeded 10. For each food supplement category sampled in the 2008 and 2009 survey, mean levels of each of the 13 EFSA priority PAH were calculated and also the mean levels of benzo[a]pyrene, PAH2, PAH4 and PAH8. The data from the whole study period were compared to the maximum levels for benzo[a]pyrene defined in Dutch food law (10 µg/kg for botanical and 2 µg/kg for regular food supplements) and, following the approach of EFSA, to all maximum levels for this PAH existing in Regulation (EC) No. 1881/2006, also because supplements are not yet regulated by this Regulation in this respect [7, 8]. Existing maximum levels for benzo[a]pyrene in Regulation (EC) No. 1881/2006 are: 1 µg/kg for baby foods, infant formulae, follow on formulae and other infant foods; 2 µg/kg for oils, fats and fish; 5 µg/kg for smoked meat and fish and some marine products and 10 µg/kg for bivalve mollusks [7].

FSA reported in 2005 levels of 15 SCF priority PAH in 207 individual food supplements from different supplement categories. Levels of 15 SCF priority PAH above the LOD were reported for each individual sample separately [9]. The FSA report did not include descriptive statistics that would aid the comparison of our results to the FSA data. We therefore calculated from the FSA data the overall lower bound mean of the benzo[a]pyrene, PAH2, PAH4 and PAH8 levels, for which we replaced each value below the LOD by 0. These parameters were also calculated for each food supplement category as defined by the FSA. We divided the category of 'Ginkgo/ Echinacaea' in two, resulting in a separate category for each of these botanicals.

Results

Results in 2003 up to and including 2007

The food supplements included in the study were grouped into main categories and subcategories based on the active ingredient or a combination of active ingredients. Table 5 shows for each subcategory the lower bound mean, median, P90, P95 and maximum benzo[a]pyrene levels obtained in 2003 up to and including 2007. Also shown for each category in this table is the percentage of samples with benzo[a]pyrene levels that exceeded the LOQ and limits that currently apply to benzo[a]pyrene in various foods in European and Dutch food law. Based on the Dutch limit for benzo[a]pyrene in botanical food supplements of 10 µg/kg, benzo[a]pyrene levels in a food supplement category were considered high in the current study when the P90 of benzo[a]pyrene levels in a category exceeded 10 µg/kg. In total, 553 (44%) of 1258 supplements contained benzo[a]pyrene levels above the LOQ and the mean benzo[a]pyrene level was 3.37 µg/kg. For several supplements with relatively high benzo[a]pyrene concentrations, estimated intake levels of this PAH were calculated by using the manufacturers' maximum recommended use level (Table 6).

Benzo[a]pyrene levels in all samples in the category of mono-ingredient botanical food supplements, which included botanicals such as *Boswellia serrata* and kelp, were below 5 µg/kg (Table 5). A multivitamin supplement with several botanicals contained 43.4 µg/kg benzo[a]pyrene which would amount to a daily intake of 229 ng of benzo[a]pyrene when used according to the maximum recommended use levels. The parameters used

for the calculation of this intake estimate are shown in Table 6 under No. 7. Supplements with green tea extracts showed the highest mean and median benzo[a]pyrene concentrations in botanicals between 2003 and 2007 (Table 5). A supplement with green tea (*Camellia sinensis*) with the highest benzo[a]pyrene level found in this period of 145 µg/kg, would provide 225 ng of benzo[a]pyrene per day when used at the maximum recommended use level. The parameters used for the calculation of this intake estimate are shown in Table 6 under No. 4. Furthermore, supplements with a tea variety called 'pu erh', which is claimed to aid in weight loss, also showed relatively high benzo[a]pyrene levels. Moreover, benzo[a]pyrene levels were relatively high in the botanicals dong quai (*Angelica sinensis*), *Ginkgo biloba*, St. John's wort (*Hypericum perforatum*) and valerian (*Valeriana officinalis*) and outside the botanical category, in propolis and pollen supplements, and natural vitamin E (d-alfa tocopherol) supplements with expiry dates before 2007. Vitamin E samples with later expiry dates displayed lower benzo[a]pyrene levels with a P90 lower than the LOQ.

Table 5 Descriptive statistics for lower bound [a] benzo[a]pyrene (BaP) levels in various supplement groups sampled in 2003 up to and including 2007 and the percentage of supplements with BaP levels above the LOD, 1, 2, 5, or 10 µg/kg

Supplement characteristics		N	Supplements (%) above limit for BaP (µg/kg)					BaP concentration (µg/kg)				
Category	Type/ ingredient [b]		>LOQ	>1	>2	>5	>10	Median	Mean	P90	P95	Max
Fatty acids	Mono-ingredient	15	20	13	7	7	0	0	0.71	1.20	3.50	8.41
	Multi-ingredient	29	24	14	7	3	0	0	0.49	1.38	2.81	5.58
	Conjugated linoleic acid	9	33	33	22	11	11	-	1.66	-	-	11.7
	Borago officinalis (borage) seed oil	7	71	29	0	0	0	-	0.61	-	-	1.11
	Linum usitatissimum (flax) seed oil	6	0	0	0	0	0	-	0	-	-	0
	Fish oil, shark liver oil	82	20	6	1	0	0	0	0.19	0.79	1.01	2.17
	Oenothera biennis (evening primrose) seed oil	47	23	17	6	4	2	0	0.75	1.28	3.84	13.9
	Triticum sp. (wheat) germ oil	21	14	5	5	5	0	0	0.51	0.60	1.00	9.12
Botanical	Mono-ingredient	39	41	26	10	0	0	0	0.61	1.86	2.35	3.73
	Multi-ingredient	128	58	51	38	20	8	1.10	2.86	9.10	11.62	33.5
	Multivitamins with and without botanicals	125	22	13	6	2	1	0	0.72	1.10	2.33	43.4
	Ayurvedic, multi-ingredient	11	55	55	18	0	0	1.06	1.23	3.21	3.89	4.56
	Chinese, mono-ingredient	8	38	38	25	25	0	-	2.41	-	-	9.59
	Chinese, multi-ingredient	10	80	80	60	20	0	3.16	2.92	5.35	5.81	6.26
	Actaea racemosa [c] (black cohosh)	4	100	75	75	50	25	-	6.29	-	-	12.5
	Allium sativum (garlic)	24	21	13	8	0	0	0	0.50	1.56	3.34	4.92
	Aloe vera	4	25	25	0	0	0	-	0.34	-	-	1.35
	Angelica sinensis (dong quai)	12	83	67	50	42	33	2.11	8.12	24.6	24.7	24.9
	Camellia sinensis (green tea)	18	83	78	72	56	44	8.13	26.6	83.1	135	145
	Camellia sinensis (pu erh tea)	3	100	100	100	100	67	-	11.6	-	-	13.8
	Echinacea spp. (coneflower)	4	50	0	0	0	0	-	0.31	-	-	0.61

Table 5 (continued)

Supplement characteristics		N	Supplements (%) above limit for BaP (µg/kg)					BaP concentration (µg/kg)				
Category	Type/ ingredient [b]		>LOQ	>1	>2	>5	>10	Median	Mean	P90	P95	Max
Botanical	*Eleutherococcus senticosus* (Siberian ginseng)	8	63	38	25	25	13	-	5.29	-	-	34.2
	Ginkgo biloba	65	77	69	55	32	25	2.73	7.48	20.2	31.3	64.4
	Glycine max (soy) protein, isoflavones	12	17	8	0	0	0	0	0.21	0.90	1.23	1.50
	Hypericum perforatum (St. John's wort)	38	82	63	39	21	11	1.60	9.87	15.7	49.5	144
	Panax ginseng (ginseng)	55	58	45	33	13	5	0.84	2.86	7.32	9.22	38.2
	Paullinia cupana (guarana)	8	50	38	0	0	0	-	0.63	-	-	1.60
	Silybum marianum [d] (milk thistle)	8	50	38	38	13	13	-	2.37	-	-	11.6
	Spirulina	8	38	38	13	0	0	-	0.69	-	-	2.44
	Uncaria tomentosa (cat's claw)	5	20	0	0	0	0	-	0.14	-	-	0.70
	Vaccinum spp. (cranberry)	5	0	0	0	0	0	-	0	-	-	0
	Valeriana officinalis (valerian)	30	73	60	50	37	17	1.90	7.87	22.2	38.4	52.6
	Zingiber officinale (ginger)	16	81	69	50	13	0	2.00	2.30	4.74	5.52	6.88
Other	Mono-ingredient without botanicals	18	6	6	6	0	0	0	0.28	0.00	0.74	4.95
	Coenzyme Q10	15	20	7	0	0	0	0	0.23	0.74	1.15	1.96
	Carotenoids	22	32	23	14	0	0	0	0.50	2.02	2.10	2.20
	Lecithin	16	0	0	0	0	0	0	0	0	0	0
	Propolis, bee pollen	10	80	70	60	30	30	4.35	6.53	16.4	17.6	18.9
	Vitamin A or D	5	0	0	0	0	0	-	0	-	-	0
	Vitamin C	20	5	0	0	0	0	0	0.05	0	0.05	0.90
	Vitamin E with expiry date before 2007	163	57	52	48	42	38	1.55	14.9	44.1	63.3	207
	Vitamin E with expiry date from 2007	37	5	5	0	0	0	0	0.06	0	0.21	1.16
	Unknown	88	56	44	35	16	11	0.75	4.07	12.1	15.5	63.4

[a] For a lower bound estimate, values for samples with PAH levels below LOD or LOQ were replaced by 0; [b] When one ingredient is listed, this substance generally represents the only active ingredient in this group; [c] syn. *Cimicifuga racemosa* ; [d] syn. *Carduus marianus*

Table 6 Examples of daily intake levels of benzo[a]pyrene (BaP) with supplements, when used according to the recommended use level as stated on the label, sampled in 2003 up to and including 2007

Supplement type	BaP (µg/kg)	Year of analysis	Maximum dose (No. of units)	Unit weight (g)	Intake BaP (ng/day)
1. Bee propolis	18.9	2003	4	0.504	38.1
2. Ginkgo biloba	64.4	2003	1	0.235	15.1
3. Green tea	29.5	2004	4	0.385	45.4
4. Green tea	145	2004	4	0.388	225
5. Green tea	39.3	2003	2	0.426	33.5
6. Multi-ingredient botanical	23	2003	2	1.21	55.6
7. Multivitamin with botanicals	43.4	2003	3	1.76	229
8. Natural vitamin E	63.4	2004	2	0.511	64.8
9. Natural vitamin E	74.6	2003	6	0.827	370
10. St John's wort	28.9	2003	2	0.571	33.0
11. St John's wort	90	2003	1	0.278	25.0
12. Valerian	17.1	2003	6	0.300	30.8
13. Valerian	21.4	2003	3	0.479	30.8
14. Valerian	29.8	2004	4	0.225	26.8

Results from 2008 up to and including 2009

In 2008 the method for benzo[a]pyrene was expanded to include more of the 16 EFSA priority PAH. Of these priority PAH, cyclopenta[cd]pyrene, benzo[j]fluoranthene and indeno[1,2,3-cd]pyrene (IcP) could not be quantified. Because indeno[1,2,3-cd]pyrene could not be determined, the PAH8 level reported in this survey lacks the levels for this PAH and was consequently referred to as PAH8(-IcP). The remaining 13 EFSA priority PAH were analyzed in 333 supplements sampled in 2008 and 2009 and the lower bound estimates for the mean benzo[*a*]pyrene, PAH2, PAH4 and PAH8(-IcP) levels were 5.33 µg/kg, 20.8 µg/kg, 33.8 µg/kg and 40.9 µg/kg, respectively. Table 7 shows for each EFSA priority PAH quantified in these 333 supplements the descriptive statistics including the lower bound mean and the percentage of the samples exceeding the LOQ and limits that currently apply to benzo[a]pyrene in various foods in European and Dutch food law. Benzo[a]pyrene levels were above the LOQ in 210 (63%) of the 333 samples. The priority PAH with the highest lower bound mean level was chrysene with a value of 15.5 µg/kg and the maximum level found for this PAH was 368 µg/kg. The priority PAH with the highest maximum level was benzo[c]fluorene with 503 µg/kg (Table 7).

Table 7 Lower bound PAH levels in food supplements (N=333) sampled in 2008 and 2009 and percentage of supplements with PAH levels exceeding the LOQ, 1, 2, 5 or 10 µg/kg

PAH	Product (%) above limit (µg/kg)					Concentration (µg/kg)				
	>LOQ	>1	>2	>5	>10	Median	Mean	P90	P95	Max
Benz[a]anthracene	29	29	29	20	11	0	5.18	11.0	24.2	188
Benzo[a]pyrene	63	52	39	23	11	1.10	5.33	10.4	17.5	150
Benzo[b]fluoranthene	57	57	45	27	15	1.60	7.80	16.2	28.0	314
Benzo[j]fluoranthene	-	-	-	-	-	-	-	-	-	-
Benzo[k]fluoranthene	65	41	29	13	7	0.80	2.93	5.90	13.2	69.1
Benzo[ghi]perylene	35	35	34	19	8	0	3.37	8.02	15.1	109
Chrysene	50	48	44	36	27	0	15.5	32.8	91.2	368
Cyclopenta[cd]pyrene	-	-	-	-	-	-	-	-	-	-
Dibenz[ah]anthracene	19	14	9	4	2	0	0.77	1.58	3.04	30.7
Dibenzo[a,e]pyrene	8	8	5	4	2	0	1.01	0	2.08	200
Dibenzo[a,h]pyrene	6	4	2	0	0	0	0.22	0	0.90	36.4
Dibenzo[a,i]pyrene	7	6	4	2	1	0	0.29	0	1.40	13.7
Dibenzo[a,l]pyrene	22	20	16	8	3	0	1.70	4.10	7.32	70.1
Indeno[1,2,3-cd]pyrene	-	-	-	-	-	-	-	-	-	-
5-methylchrysene	24	22	17	10	7	0	3.22	4.58	22.5	94.8
Benzo[c]fluorene	21	19	16	8	7	0	5.08	3.58	12.6	503

For each subcategory of supplements sampled in 2008 and 2009, mean benzo[a]pyrene, PAH2, PAH4 and PAH8(-IcP) levels were computed including the percentage of samples with benzo[a]pyrene levels that exceeded the LOQ and limits for benzo[a]pyrene in Dutch and European food law (Table 8).

The results obtained in 2008 and 2009 will be discussed by focusing on the PAH4 levels. Figure 1 presents the minimum, median and maximum PAH4 levels of the different food supplement categories determined in the 2008 and 2009 survey. High PAH4 levels were defined for the purpose of the current study as a mean above 40 µg/kg of PAH4. This definition was based on the Dutch limit of 10 µg/kg for benzo[a]pyrene, which is one of the PAH4, in botanical food supplements. For several individual supplements of interest, reference is made in the text to specific entries in Table 9. This table presents for a total of 20 supplements sampled in 2008 and 2009 with relatively high PAH levels, the estimated daily intake levels of benzo[a]pyrene, PAH2, PAH4 and PAH8(-IcP), when used at the manufacturers' maximum recommended dose level.

Table 8 Percentage of supplements with benzo[a]pyrene levels exceeding the LOQ, 1, 2, 5, or 10 µg/kg and lower bound [a] mean BaP, PAH2, PAH4 and PAH8 (-IcP) [b] levels in 333 supplements sampled in 2008 and 2009

Supplement characteristics		N	Product (%) above limit for BaP (µg/kg)					Mean level (µg/kg)			
Category	Type/ ingredient [c]		>LOQ	>1	>2	>5	>10	BaP	PAH2	PAH4	PAH8 (-IcP)
Fatty acids	Miscellaneous	5	40	40	40	20	0	1.94	2.78	7.50	10.5
	Oenothera biennis (evening primrose) seed oil	7	29	29	29	0	0	0.87	7.24	9.36	9.80
	Fish liver oil	4	25	25	0	0	0	0.30	2.18	2.58	2.73
Botanical	Mono-ingredient	30	43	33	20	7	0	1.06	4.62	7.23	8.79
	Multi-ingredient	111	68	53	43	21	9	3.69	17.5	30.0	35.6
	Multivitamins with botanicals	12	58	58	25	8	8	2.93	13.7	20.3	25.3
	Ayurvedic, multi-ingredient	12	67	58	25	17	0	2.03	9.31	11.9	14.6
	Chinese, multi-ingredient	11	27	18	18	18	9	4.41	17.0	38.0	42.8
	Actaea racemosa [d] (black cohosh)	7	86	43	14	14	0	1.51	2.90	7.01	9.67
	Angelica sinensis (dong quai)	4	75	75	50	25	0	3.00	9.78	15.8	20.2
	Camellia sinensis (green tea)	18	89	72	50	28	6	3.59	8.44	13.5	19.1
	Ginkgo biloba	22	59	41	27	18	9	4.32	31.0	46.7	53.4
	Glycine max (soy) protein, isoflavones	9	22	11	0	0	0	0.27	1.48	2.02	2.64
	Hypericum perforatum (St. John's wort)	14	86	79	71	57	43	6.89	22.6	40.4	55.9
	Panax ginseng (ginseng)	8	50	38	13	0	0	0.71	0.71	1.85	2.78
	Resveratrol[e]	7	100	100	86	71	43	36.8	111	183	227
	Resveratrol, multi-ingredient	14	93	86	86	64	50	36.1	102	152	190
	Rhodiola rosea (golden root)	5	80	60	40	20	0	2.74	6.64	12.4	16.1
	Vaccinum spp. (cranberry)	5	20	20	20	0	0	0.60	0.60	1.16	1.42
	Valeriana officinalis (valerian)	8	63	63	63	50	13	4.64	8.81	20.2	24.1
Other	Miscellaneous, without botanicals	9	33	33	11	11	11	2.14	5.37	7.87	11.8
	Propolis, pollen, royal jelly	11	91	91	64	45	27	7.63	66.0	99.7	107

[a] For a lower bound estimate, values for samples with PAH levels below LOD or LOQ were replaced by 0; [b] (-IcP): indeno[1,2,3-cd]pyrene not analyzed; [c] When one ingredient is listed, this substance generally represents the only active ingredient in this group; [d] Syn. *Cimicifuga racemosa* ; [e] In 6 out of 7 supplements, resveratrol was possibly derived from *Polygonum cuspidatum*-extract

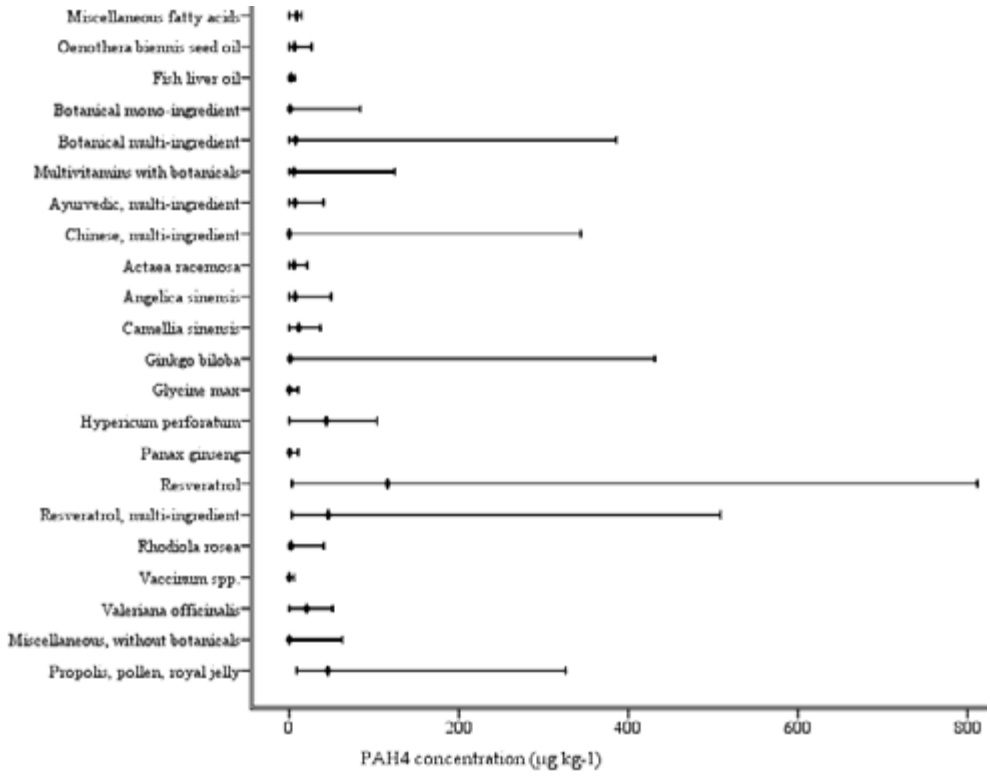

Figure 1 Minimum, median (♦) and maximum levels of PAH4 (µg/kg) in different categories of food supplements sampled in 2008 and 2009

Mono-ingredient botanical supplements including botanicals such as cat's claw (*Uncaria tomentosa*), *Eleutherococcus senticosus, Echinacaea* or red clover, showed relatively low PAH4 levels except for a supplement with brown marine algae with a PAH4 level of 84 µg/kg (Table 8). PAH4 levels of 17 multi-ingredient botanical supplements (n=111) were higher than 40 µg/kg. Estimated daily PAH4 intakes for four of these supplements when used at the maximum dose level recommended by the manufacturer, ranged from 69 to 170 ng per day. Intake estimates for other PAH indicators and the parameters used to calculate these, are shown in Table 9 under Nos. 7, 8, 9 and 10. A multivitamin supplement with several botanicals such as valerian, golden root and St. John's wort was found to contain 125 µg/kg PAH4 and 23 µg/kg benzo[a]pyrene. According to the producer the high PAH levels were caused by the use of a batch of St. John's wort containing 94 µg/kg benzo[a]pyrene. Use of this supplement at the highest recommended dose level would result in a PAH4 exposure of 99 ng per day. Additional PAH exposure estimates and the parameters used to calculate these for this supplement are shown in Table 9 under No. 16.

In Chinese multi-ingredient botanical supplements, the mean PAH4 level of 38 µg/kg resulted mainly from a sample with 340 µg/kg of PAH4, while the median PAH4 level was below the LOQ (Figure 1, Table 8). In mono-ingredient botanical supplements with black cohosh (*Actaea racemosa*), dong quai, green tea, ginseng (*Panax ginseng*) or valerian, mean benzo[a]pyrene levels were lower in the 2008 and 2009 survey than in the survey from 2003 up to and including 2007 (Table 5 and 8). Furthermore, mean PAH4 levels in these categories from 2008 and 2009 remained below 40 µg/kg (Table 8).

Table 9 Examples of daily intake levels of benzo[a]pyrene (BaP), PAH2, PAH4, PAH8 (-IcP) [a] with supplements when used according to the recommended use level as stated on the label; samples from 2008 and 2009

Food supplement sample	Maximum dose (No. of units)	Unit weight (g)	Intake (ng/day)			
			BaP	PAH2	PAH4	PAH8 (-IcP)
1. Chitosan and vitamin C	2	0.657	22	57	82	127
2. *Ginkgo biloba*	3	0.341	14	258	394	436
3. *Ginkgo biloba*	1	0.413	24	112	178	211
4. *Ginkgo biloba* leaf extract and herb, taurine	1	0.492	5	41	64	74
5. *Hypericum perforatum* extract	1	0.369	4	21	38	51
6. *Hypericum perforatum* extract	2	0.268	8	24	44	57
7. Multi-ingredient botanical	4	0.521	31	68	130	174
8. Multi-ingredient botanical	4	0.610	47	130	170	229
9. Multi-ingredient botanical	1	0.622	15	40	69	91
10. Multi-ingredient botanical	1	0.409	4	120	152	169
11. Multi-ingredient botanical with resveratrol from *Polygonum cuspidatum*	2	0.504	39	39	56	93
12. Multi-ingredient botanical with resveratrol from *Polygonum cuspidatum*	1	0.56	40	156	192	237
13. Multi-ingredient botanical with resveratrol	3	0.633	171	400	705	934
14. Multi-ingredient botanical with resveratrol	2	0.28	38	242	283	329
15. Multivitamin with botanicals	2	1.31	43	282	405	480
16. Multivitamin with botanicals	2	0.397	18	48	99	130
17. Multivitamin with resveratrol from *Polygonum cuspidatum*	1	0.396	23	51	95	131
18. *Polygonum cuspidatum* (with resveratrol)	1	0.394	59	176	320	398
19. *Polygonum cuspidatum* root extract (with resveratrol)	4	1.011	76	211	265	378
20. *Polygonum cuspidatum* root extract (with resveratrol)	3	0.724	146	146	278	346

[a] (-IcP): indeno[1,2,3-cd]pyrene not analyzed

The mean PAH4 level in *Ginkgo biloba* supplements analyzed in 2008 and 2009 (n=22) was relatively high (47 µg/kg) but the median PAH4 level (1.1 µg/kg) was considerably lower (Figure 1, Table 8). The high mean PAH4 level mainly resulted from 5 *Ginkgo biloba* supplements with PAH4 levels above 40 µg/kg and 4 of these contained more than 100 µg/kg PAH4. Use of the *Ginkgo biloba* supplement with the highest PAH4 level of 430 µg/kg at the maximum recommended dose level would result in a PAH4 intake of 178 ng per day. Additional intake estimates for other PAH indicators and the parameters used to calculate these are shown in Table 9 under No. 3. St. John's wort (*Hypericum perforatum*) samples from 2008 and 2009 contained relatively high PAH levels (Table 8). Compared to the *Ginkgo biloba* supplements sampled in 2008 and 2009, PAH contamination of St. John's wort samples from the same period was more evenly distributed over the samples and the mean PAH4 level of 40.4 µg/kg was close to the median PAH4 level of 43.7 µg/kg (Figure 1, Table 8).

Mono-ingredient supplements with resveratrol showed the highest mean PAH4 level in 2008 and 2009. A supplement only containing resveratrol from *Polygonum cuspidatum* root showed the highest benzo[a]pyrene level (150 µg/kg), PAH4 level (812 µg/kg) and PAH8(-lcP) level (1009 µg/kg) found during the whole study period from 2003 up to and including 2009. In addition, the benzo[c]fluorene level (117 µg/kg) was also high. Use at the maximum recommended dose level of this supplement would result in a PAH4 intake of 320 ng per day. Intake estimates for other PAHs and the parameters used to calculate these for this supplement are presented in Table 9 under No. 18. A multi-ingredient supplement with resveratrol, wine grape extract and quercetin contained 474 µg/kg benzo[c]fluorene and another resveratrol supplement from the same distributor, which also included wine grape extract (OPC), green tea polyphenols and rutin, contained 503 µg/kg benzo[c]fluorene. These benzo[c]fluorene levels were the highest levels found for any PAH in this survey and were comparable to the PAH8(-lcP) levels in these supplements (423 and 593 µg/kg, respectively). PAH4 exposure estimates for these supplements at the maximum recommended dose level were 192 ng and 283 ng per day, respectively. Additionally, benzo[a]pyrene, PAH2 and PAH8 intake estimates for these supplements and the parameters used to calculate these, are shown in Table 9 under Nos. 12 and 14, respectively. Another multi-ingredient botanical with resveratrol contained 371 µg/kg PAH4 and use of this product at the highest proposed dose level would result in a PAH4 intake level of 705 ng per day. Additional PAH intake estimates and the parameters used to calculate these for this supplement are presented in Table 9 under No. 13.

PAH levels in the category of miscellaneous supplements without botanicals shown in Table 8 were low. However, a supplement with chitosan and vitamin C from this category contained 63 µg/kg PAH4. Use of this supplement would result in 82 ng PAH4 per day when used at the maximum recommended dose level. Additional PAH intake estimates and the parameters used to calculate these for this supplement are presented in Table 9 under No. 1. Furthermore, PAH4 levels in propolis supplements were overall relatively high (Figure 1).

Table 10 shows for each of the 13 EFSA priority PAH determined in this survey the mean PAH level in each subcategory of food supplements sampled in 2008 and 2009. Supplements with resveratrol (alone or in combination with other botanicals) showed for 11 of these PAH the highest mean levels. The highest mean dibenzo[ae]pyrene and dibenzo[ah]pyrene levels were found in Chinese botanical supplements.

Table 10 Lower bound mean levels of 13 PAH in 333 supplements sampled in 2008 and 2009

Supplement characteristics		N	Mean PAH concentration (µg/kg) [b]												
Category	Type/ ingredient [a]		BaP	BgP	BaA	BbF	BkF	CHR	DhA	DeP	DhP	DiP	DlP	5MC	BcL
Fatty acids	Miscellaneous	5	1.94	1.40	0.92	3.80	1.44	0.84	0.14	0	0	0	0.62	0.98	0
	Oenothera biennis seed oil	7	0.87	0	1.20	0.91	0.44	6.37	0	0	0	0.36	0.14	0.24	0
	Fish liver oil	4	0.30	0	0	0.40	0.15	1.88	0	0	0	0	0	0.68	0
Botanical	Mono-ingredient	30	1.06	0.84	0.94	1.67	0.59	3.56	0.12	0	0	0.05	0.45	0.57	0.34
	Multi-ingredient	111	3.69	2.57	4.33	8.16	2.54	13.8	0.54	0.40	0.05	0.10	1.08	2.20	2.04
	Multivitamin, with botanicals	12	2.93	2.86	3.14	3.53	1.74	10.7	0.36	0	0	0.07	0.34	0.20	1.41
	Ayurvedic, multi-ingredient	12	2.03	1.44	0.63	1.98	1.02	7.28	0.21	0.30	0	0.12	0.37	10.2	2.42
	Chinese, multi-ingredient	11	4.41	2.20	9.04	12.0	1.04	12.6	1.53	18.1	3.31	1.23	6.88	7.33	1.52
	Actaea racemosa [c] (black cohosh)	7	1.51	1.61	0.81	3.30	1.04	1.39	0	0	0	0	0.46	0	0.41
	Angelica sinensis (dong quai)	4	3.00	2.43	1.03	5.03	1.95	6.78	0	0	0.23	0	1.25	0.48	9.63
	Camellia sinensis (green tea)	18	3.59	3.61	1.62	3.47	1.60	4.86	0.39	0.07	0.12	0.08	1.55	1.23	0.73
	Ginkgo biloba	22	4.32	2.40	7.18	8.5	3.82	26.7	0.53	0.35	0.13	0.37	0.20	1.65	1.23
	Glycine max (soy) protein, isoflavones	9	0.27	0	0	0.54	0.28	1.21	0.34	0	0	0	0	0	0
	Hypericum perforatum (St. John's wort)	14	6.89	6.78	7.8	9.9	5.78	15.7	2.91	0.41	0.13	0.14	2.19	2.46	3.66
	Panax ginseng (ginseng)	8	0.71	0.34	0.53	0.61	0.49	0	0.10	0	0	0	0	2.88	0.20
	Resveratrol [d]	7	36.8	21.8	28.4	43.0	16.9	74.4	5.34	3.73	1.06	0.77	11.07	26.8	17.9
	Resveratrol, multi-ingredient	14	36.1	19.3	15.2	35.5	14.7	65.4	3.66	1.74	0.94	3.41	11.5	6.99	76.2
	Rhodiola rosea (golden root)	5	2.74	1.90	2.94	2.80	1.38	3.90	0.44	0	0.36	0	0.26	0	0.54
	Vaccinum spp. (cranberry)	5	0.60	0	0	0.56	0.26	0	0	0	0	0	0	0	0
	Valeriana officinalis (valerian)	8	4.64	1.44	6.90	4.45	2.46	4.18	0	0	0	0	1.29	0.49	0.36
Other	Miscellaneous, without botanicals	9	2.14	3.12	0	2.50	0.83	3.22	0	1.41	0	0.30	1.14	2.18	0
	Propolis, pollen, royal jelly	11	7.63	1.96	24.2	9.47	4.08	58.4	1.18	0.84	0	0	1.16	15.4	5.37

[a] When one ingredient is listed, this substance generally represents the only active ingredient in this group

[b] See table 1 for the abbreviations used for the 13 EFSA priority PAH determined in this survey

[c] syn. *Cimicifuga racemosa*

[d] 6 out of 8 supplements labeled a *Polygonum cuspidatum*-extract as the source of resveratrol

Table 11 Lower bound mean BaP, PAH2, PAH4 and PAH8 levels in food supplements calculated from results reported by the FSA in 2005 [a]

Supplement characteristics				Mean concentration (µg/kg)			
Main category	FSA category [b]	Matching VWA category [c]	N	BaP	PAH2	PAH4	PAH8 [d]
Fatty acids	Animal oils	-	3	0.28	0.66	1.67	2.58
	Fish oil	Fish oil	9	0.08	0.23	0.37	0.59
	Combination fish oil	Multi-ingredient	8	0.16	1.0	1.6	1.9
	Plant oils [e]	Mono-ingredient	15	0.21	1.12	1.75	2.22
Botanical	Plant extracts [f]	Mono-ingredient	26	6.27	26.5	48.1	61.0
	Garlic	*Allium sativum*	10	0.47	7.03	12.1	13.6
	Echinacea	*Echinacea* spp.	7	0.31	2.41	3.44	4.27
	Ginkgo	*Ginkgo biloba*	8	4.55	37.9	57.3	66.9
	Ginseng	*Panax ginseng*, *Eleutherococcus senticosus*	15	3.96	11.4	21.2	29.3
	Other aquatic products	Spirulina; miscellaneous, without botanicals	8	6.51	55.0	91.4	112
Other	Antioxidant	Miscellaneous, without botanicals	10	0.28	2.2	3.5	4.3
	Single vitamin	Mono-ingredient without botanicals, vitamin C or E	7	0.05	0.58	0.95	1.10
	Multivitamin	Miscellaneous, without botanicals	9	0.57	1.39	2.38	3.37
	Multivitamin – child	Miscellaneous, without botanicals	10	0	0.05	0.05	0.05
	Minerals	Mono-ingredient, without botanicals	17	0	0.10	0.16	0.20
	Calcium	Mono-ingredient, without botanicals	8	0.04	0.16	0.25	0.38
	Iron	Mono-ingredient, without botanicals	10	0.04	0.19	0.30	0.40
	Zinc	Mono-ingredient, without botanicals	7	0.33	1.66	2.77	3.42
	Bee products	Propolis, pollen, royal jelly	12	4.74	42.0	61.8	73.8
	Glucosamine	Mono-ingredient, without botanicals	8	0.04	0.33	0.51	0.65

a) Lower bound estimates for the mean BaP, PAH2, PAH4 and PAH8 levels were calculated from results of the FSA survey on PAHs in Dietary supplements from 2005 [9]. The lower bound estimate was calculated by replacing values reported as lower than the LOD or LOQ, by 0.

b) The FSA subcategories were taken from the FSA report [9], the FSA category Ginkgo/ Echinacea was separated into one for each botanical however.

c) Matching categories found in tables 5, 8 and 10

d) Including indeno[1,2,3-cd]pyrene

e) Including samples with *Borago officinalis*, *Linum usitatissimum* and *Oenothera biennis* seed oil, CLA and others.

f) Including samples with *Actaea racemosa*, *Aloe vera*, *Angelica sinensis*, *Camellia sinensis*, *Glycine max*, *Hypericum perforatum*, *Silybum marianum*, *Uncaria tomentosa*, *Vaccinum spp.*, *Valeriana officinalis* and others

We calculated from FSA data on 15 SCF priority PAH in 207 individual food supplements [9] the lower bound mean benzo[a]pyrene, PAH2, PAH4 and PAH8 levels which amounted to respectively, 1.90, 11.0, 18.3, and 22.7 µg/kg. These parameters were also calculated for each category of food supplements used by the FSA (Table 11). The relatively high PAH levels in the FSA category 'plant extracts' resulted mainly from individual supplements with spirulina, soy isoflavones, green tea, black cohosh, dong quai, St. John's wort and red clover. The mean benzo[a]pyrene level of ginseng supplements (Table 11) matched best our findings for this botanical from 2003 up to and including 2007 (Table 5). A spirulina supplement with a PAH4 level of 660 µg/kg and a PAH8 level of 789 µg/kg contributed considerably to he relatively high mean PAH level in the FSA category 'other aquatic products' (Table 11). From the maximum recommended dose level reported by the FSA we calculated a daily PAH8 intake level of 4120 ng/day for this supplement.

Discussion

Chapter 7 of this thesis presents the levels of benzo[a]pyrene and several of the priority PAH defined in 2008 by EFSA in food supplements containing botanicals and other ingredients on the Dutch market. From 2003 up to and including 2007, benzo[a]pyrene was present above the LOQ in 553 (44%) of 1258 supplements and the mean benzo[a]pyrene level was 3.37 µg/kg. In the 2008 and 2009 survey, 210 (63%) of 333 food supplements showed benzo[a]pyrene levels above the LOQ and the lower bound mean benzo[a]pyrene level was 5.33 µg/kg. In 2008 and 2009, besides benzo[a]pyrene 12 other EFSA priority PAH were also determined and the lower bound mean PAH2, PAH4 and PAH8 (minus indeno[1, 2, 3-cd]pyrene; IcP) levels were 20.8, 33.8 µg/kg and 40.9 µg/kg, respectively. In this period, mono-ingredient supplements with resveratrol, St. John's wort, *Ginkgo biloba* and propolis showed the highest mean PAH levels. Prior to 2008, the highest mean benzo[a]pyrene levels were found in mono-ingredient supplements with dong quai, green tea, *Ginkgo biloba*, St. John's wort, valerian, propolis and pollen, and in natural vitamin E supplements with expiry dates before 2007.

PAH levels in natural vitamin E supplements with expiry dates after 2007 were markedly lower in natural vitamin E supplements with earlier expiry dates (Table 5). In addition, PAH levels in supplements with green tea, dong quai, green tea, valerian and ginseng were lower in 2008 and 2009 relative to the previous period. These findings might reflect frequent monitoring of PAH levels in food supplements by the VWA in combination with increased awareness of manufacturers. However, PAH levels in supplements with St. John's wort, *Ginkgo biloba* and propolis remained high and supplements with resveratrol appeared as a new category with relatively high PAH levels. High PAH levels in propolis have also been reported in several European Member States [9, 11, 12]. High PAH levels in natural vitamin E of the three major manufacturers in the world were described in a US patent from 1994 [15]. The reported benzo[a]pyrene levels in the patent of 28, 30 and 106 µg/kg were in line with our results for vitamin E food supplements with expiry dates before 2007 (Table 5) and our previous results [5]. Higher benzo[a]pyrene levels were reported for 2 different natural vitamin E products (mixed tocopherols) from 2 Chinese manufacturers, which were 469.5 ± 36.8 µg/kg and 315.0 ± 9.5 µg/kg [16]. PAH8 levels in these two products were 2700 and 1500 µg/kg (rounded of to the nearest 100), respectively.

Our results were in agreement with a report of the FSA of 15 SCF priority PAH in a wide range of food supplements (n=207) sampled in 2004 [9]. The FSA data also showed relatively high mean PAH levels in *Ginkgo biloba* supplements and bee products (high levels were limited to propolis supplements). The lower bound mean benzo[a]pyrene, PAH2, PAH4, PAH8 levels (1.90, 11.0, 18.3, and 22.7 µg/kg, respectively) calculated from the FSA data were lower than our findings, which might be explained by differences in sampling strategies. With respect to benzo[a]pyrene, the levels reported by the FSA fitted best the data obtained by us from 2003 up to and including 2007 (Table 5). Danyi et al. determined the 16 EFSA priority PAH in 20 botanical mono-ingredient food supplements of which St. John's wort showed the highest PAH levels. Benzo[c]fluorene was found in two supplements [4]. The results were within the range found in our survey but the number of samples was too low for a meaningful comparison. Danyi et al. also briefly addressed possible mechanisms by which botanical materials could become contaminated with PAH which included direct atmospheric deposition on plant surfaces, absorption from soil and drying practices during manufacturing [4]. JECFA recommended that contact of food with combustion gases should be minimized [2, 17]. Direct drying procedures that use combustion gasses as the drying gas that comes in contact with the foods increased PAH contamination by 3- to 10-fold, dependent on the fuel used [17]. The Codex Alimentarius code of practice for the reduction of contamination of food with PAH from smoking and direct drying processes advised therefore against the use of diesel oil, rubber, tyres or waste oil as fuel [17].

EFSA reported in 2008 in an Opinion of the Panel on Contaminants in the Food Chain on PAH in food, lower and upper bound mean benzo[a]pyrene, PAH2, PAH4, PAH8 levels derived from data of 283 food supplements submitted to EFSA by five European countries including the UK [10]. The lower bound mean levels for these parameters were 2.78, 12.8, 23.7 and 30.0 µg/kg respectively [3]. We found in 2008 and 2009 higher values for these parameters, which amounted to respectively 5.33, 20.8, 33.8 and 40.9 µg/kg. Our sampling protocol was targeted at supplements with relatively high PAH levels whereas the data submitted to EFSA were obtained by both targeted and random sampling. Mean PAH8 levels in food supplements came second in the EFSA Opinion after levels in dried tea samples with a lower bound mean of 61.0 µg/kg PAH8 [3]. The highest individual PAH level reported for foods tested for all 15 SCF priority PAH was 1064 µg/kg benz[a]anthracene in tinned sprats. According to EFSA this value was possibly an aberration, however. The second and third highest individual PAH level (690 µg/kg of benzo[b]fluoranthene and 590 µg/kg of chrysene, respectively) were found in food supplements [3]. In the category of supplements, a black tea supplement showed the highest combined PAH concentrations and a supplement with spirulina and one with propolis extract showed the second and third highest concentrations, respectively [10]. In our study, the highest and second highest individual PAH levels were found in food supplements with resveratrol amounting to 503 and 474 µg/kg benzo[c]fluorene, respectively. EFSA received data on benzo[c]fluorene in only 24 food supplements and 9 (37.5%) of these contained this PAH with a maximum of 9.6 µg/kg [3]. We tested 333 supplements for benzo[c]fluorene and 70 (21%) contained this PAH with a maximum of 503 µg/kg (Table 7).

With respect to using lower or upper bound PAH levels, EFSA concluded that overall there was a very limited impact of using the lower or the upper bound, but the choice for the upper bound would represent a small

but real overestimation [3]. In the current study we chose to report the lower bound PAH levels in order to avoid overestimation. EFSA calculated the lower bound by replacing values below the LOD by 0. However, conflicting information was provided by EFSA in the Opinion on PAH in food on how values reported by Member States to be below the LOQ were treated. The first method provided was replacing values below the LOQ by 0, and a second was replacing values below the LOQ by the value for the LOD for each individual PAH [3]. In the current study we calculated the lower bound PAH levels by replacing both the values below the LOD and the LOQ by 0. This approach is therefore likely to result in an underestimation of actual PAH levels in food supplements.

EFSA calculated in their Opinion on PAH in food margins of exposure (MOEs) for benzo[a]pyrene, PAH2, PAH4 and PAH8 by dividing the lowest Benchmark Dose Lower Confidence Limit associated with an extra 10% cancer risk relative to background incidence (BMDL10) by the estimated mean exposure to these PAH in the European Union (EU) [3]. From the estimated mean dietary exposure to PAH8 in the EU of 28.8 ng per kg body weight per day (ng/kg bw/day) and the lowest BMDL10 for PAH8 identified by EFSA of 0.49 mg/kg bw/day, an MOE of 17,000 applicable to average consumers was calculated [3]. Similarly, for the 97.5th percentile of the estimated dietary exposure to PAH8 in the EU of 51.3 ng/kg bw/day, an MOE of 9600 applicable to high level consumers was derived [3]. EFSA concluded that the MOEs calculated for average consumers indicated a low concern for consumer health but that the MOEs for high level consumers are close to or less than 10,000 which indicates a potential concern for consumer health and a possible need for risk management action [3, 18].

In the current study, the highest estimated daily exposure to PAH8(-IcP) of 934 ng was calculated for a multi-ingredient botanical food supplement with resveratrol (No. 13 from Table 9). Assuming a body weight of 60 kg, the exposure to PAH8(-IcP) with this supplement used at the maximum recommended dose level would amount to 15.6 ng/kg bw/day. From this value and the lowest BMDL10 value for PAH8 identified as acceptable by EFSA of 0.49 mg/kg bw/day [3], an MOE of 31,500 (rounded of to the nearest 100) was calculated. Similarly, MOEs calculated for the daily exposure to benzo[a]pyrene, PAH2 and PAH4 from this supplement at the recommended dose level by using BMDL10 values of respectively 0.07, 0.17 and 0.34 mg/kg bw/day [3], were 24,600; 25,500 and 28,900, respectively. Although these MOEs were higher than 10,000, the estimated daily benzo[a]pyrene intake of 2.85 ng/kg bw/day with this supplement at the recommended dose level exceeded the virtual safe dose (VSD) for all dietary PAH expressed as 0.5 ng/kg bw/day of benzo[a]pyrene established in 2001 by the Dutch National Institute for Public Health and the Environment (RIVM) using benzo[a]pyrene as an indicator for the occurrence and carcinogenic potency of dietary PAH. The VSD was based on a rat study with coal tar mixture administered by oral gavage [19]. The estimated PAH8 intake from this particular supplement would amount to more than 50% of the estimated mean dietary exposure to PAH8 in the European Union calculated by EFSA and 30% of the 97.5th percentile of estimated dietary exposure to PAH8 in the European Union derived by EFSA [3].

Relatively high estimated PAH intakes with individual food supplements containing high PAH levels have been reported in other studies as well [9, 11]. In the FSA study [9], a spirulina supplement used at the maximum recommended dose level would provide 68.7 ng/kg bw/day of PAH8, which would result in an MOE of 7100 calculated by using a BMDL10 for PAH8 of 0.49 mg/kg bw/day. In a study on PAH in propolis on the Italian

market, high estimated PAH8 intakes were derived for several propolis products [11]. For a sample of raw propolis, a daily PAH8 intake of 46.7 ng/kg bw/day was estimated for a 60 kg adult by assuming a daily dosage of 200 mg [11]. From this estimated PAH8 intake an MOE of 10,500 could be calculated. However, the daily dosage used to estimate the PAH8 exposure was rather low. Our study included a propolis supplement with a maximum recommended dose level of 2 g per day (No. 1 of Table 6). Recommended dosages of 3 g propolis per day were also found on the Internet [11]. When a recommended dose level of 2 g would be assumed for this particular raw propolis sample, the MOE would be tenfold lower thus resulting in an MOE below 10,000 [11].

EFSA also presented data on the contributions of individual food categories to PAH exposure of consumers of these foods. These contributions were calculated by first collecting for each food category the mean consumption of consumers only in each Member State and then by multiplying the median of these by the mean contamination [3]. The contributions thus obtained ranged from 30 ng PAH8 per day for cheese up to 421 ng per day for seafood and seafood products [3]. In our 2008 and 2009 survey, the range of the estimated daily PAH8(-IcP) intakes at the recommended dose level of 20 supplements with relatively high PAH levels was 51 up to 934 ng per day (Table 9). Use at the recommended dose level of three of these supplements would result in PAH8(-IcP) exposure exceeding the highest contribution of a food category to PAH8 exposure of consumers [3]. This shows that individual food supplements can contribute significantly to the daily PAH exposure of consumers. Collectively, the PAH intake from food supplements will be less substantial however. From the mean PAH8(-IcP) level of 40.9 µg/kg found in our 2008 and 2009 survey and an assumed daily dosage of 1 g, a daily PAH8(-IcP) intake of 41 ng per day, equivalent to 0.7 ng/kg bw/day for a 60 kg adult, could be estimated. This intake estimate of PAH8(-IcP) would amount to 2.4% of the mean level dietary exposure to PAH8 in the EU and 1.3% of the high level dietary exposure [3]. Furthermore, the PAH8(-IcP) intake of 41 ng per day estimated above for food supplements (mainly with botanical ingredients) was comparable to contributions from cheese (30 ng PAH8 per day) and sugar and sugar products including chocolate (39 ng PAH8 per day) to PAH exposure of consumers [3].

Maximum levels for contaminants in European Food law should be set at a level, which is as low as reasonably achievable (ALARA) when these contaminants are considered to be genotoxic carcinogens [7]. It was interesting to note that overall in 192 (58%) of the supplements in the 2008 and 2009 survey (n=333), PAH4 levels were lower than 10 µg/kg and in 99 (30% of the total) of these PAH4 levels were below the LOQ. Furthermore, in all subcategories of food supplements identified in this survey there were supplements that contained no quantifiable or relatively low levels (here defined as PAH4 level below 10 µg/kg) of PAH. This suggests that contamination of food supplements with PAHs is avoidable.

In summary, it can be concluded that use at maximum recommended dose levels of individual food supplements, especially those with botanicals or propolis, can contribute significantly to PAH exposure, whereas collectively, PAH intake resulting from food supplement use will be at the lower end of the range of contributions of main food groups to PAH exposure of consumers of these foods. Efforts aiming at regular control and reduction of PAH levels in food supplements may prove a way forward to further reduce the intake of these genotoxic carcinogens from food.

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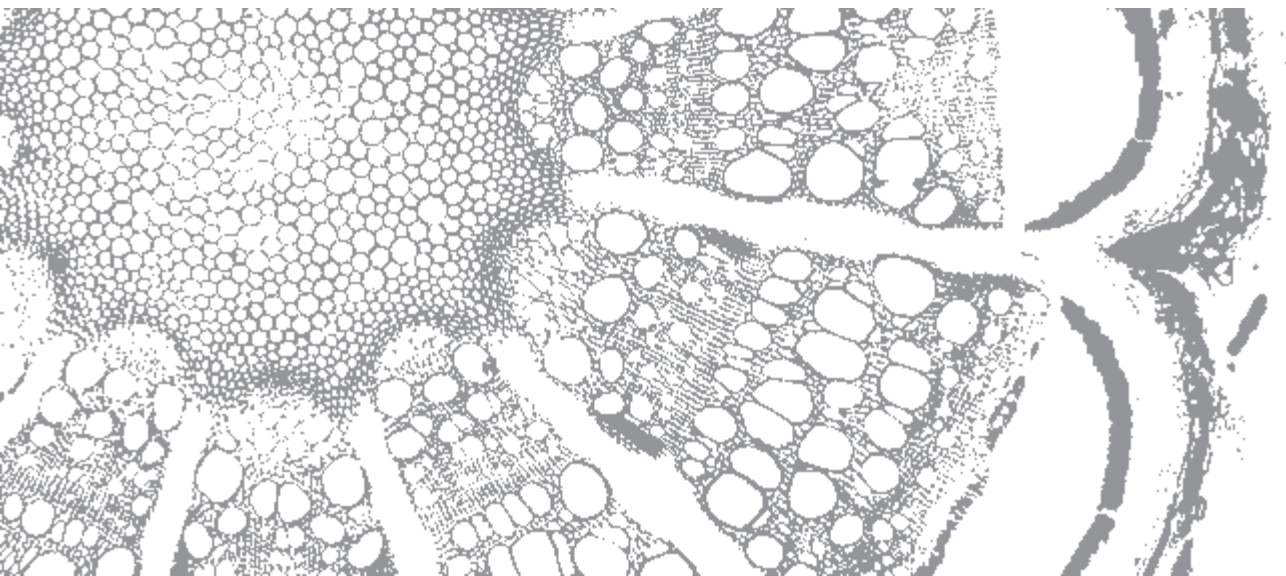
CHAPTER
8

Summary and general discussion



Background

In the last two decades it became increasingly recognized that the availability and use of botanicals outside the regulatory framework for medicinal products was increasing in the Netherlands and in other Member States of the European Union and that also certain acutely toxic herbs and herbs with delayed toxic effects were being brought on the market [1, 2]. In 2004, the European Food Safety Authority (EFSA) considered in a position paper on this topic that, because food supplements with botanical materials and preparations were gaining in popularity, safety concerns linked to these products also became more prominent. The safety concerns linked to botanicals and botanical preparations essentially relate to i) the presence of naturally occurring toxic substances in plants, ii) the intentional addition or accidental occurrence of toxic contaminants and iii) interactions of botanical ingredients with active ingredients from medicinal products (herbal food-drug interactions) [2].

The presence of naturally occurring toxic substances in botanicals and botanical preparations is an important reason for safety concerns and several of these concerns have been addressed in the Netherlands by the Commodities Act Decree 'Herbal preparations'. The Decree entered into force in 2001 and applies to the use of plants and fungi in food and non-food commodities. Herbal preparations are defined by this Decree as 'herbal substances, subjected to treatment or not, including herbal extracts, which are intended to be used by humans'. In addition, 'herbal substances' are defined as 'substances that are composed of plant material'. The Decree limits the presence of several naturally occurring toxic substances, such as for example aristolochic acids, in herbal preparations and it bans the use of certain toxic plants and fungi in herbal preparations [1]. Outside the regulatory context, the terms 'herbal preparation' and 'botanical preparation' or 'botanical' can be used interchangeably.

A second reason for concerns about the safety of botanicals and botanical preparations relates to the intentional addition or accidental occurrence of a wide range of toxic contaminants. Contamination with lead, mercury and arsenic of herbal preparations has been described to occur in several Asian traditional medicine systems resulting in poisonings worldwide [3]. In the Indian traditional medicine system Ayurveda, Traditional Tibetan Medicine (TTM) and in Traditional Chinese Medicine (TCM), the metals mercury and lead and the metalloid arsenic are deliberately added to herbal preparations for therapeutic reasons. Metals and metalloids such as mercury and arsenic can exist in different defined chemical species and the toxicity of these species can vary significantly. In order to estimate the risks related to exposure to metals and metalloids from food it is important to take into account the chemical species in which these metals and metalloids exist. Furthermore, contamination of herbal preparations with polycyclic aromatic hydrocarbons (PAH) occurs. This may be the result of inadequate processing procedures such as drying practices where raw plant material comes in direct contact with combustion gasses. Contamination may also result from environmental deposition and these environmental PAH may be concentrated by the extraction steps applied in the production process of the botanical preparation, resulting in the past in for instance natural vitamin E samples that were highly PAH contaminated [4-6]. Several PAH such as benzo[a]pyrene, are genotoxic and carcinogenic. PAH were evaluated by several risk assessment bodies including EFSA, which identified 16 priority PAH. EFSA concluded that 8 of these 16 EFSA priority PAH

for which oral carcinogenicity data are available (PAH8) could be used as indicator of the carcinogenic potency of PAH in food. According to EFSA, the most suitable indicators of PAH in food with regards to both occurrence and toxicity are currently a combination of 4 compounds of the PAH8 (referred to as PAH4) or PAH8 itself [7].

Finally, toxicological concern is raised by the potential of certain botanicals and botanical preparations to interact with conventional medicines leading to serious adverse effects. St. John's wort is a well-known example of a herb for which serious herb-drug interactions have been described and the mechanism of action is discussed in some detail in Chapter 3 of this thesis. Risks of herb-drug interactions were however not discussed further in this thesis.

The aim of the present thesis was to review the toxicity of selected herbal preparations, to investigate the presence and actual levels of selected contaminants and naturally-occurring toxic substances in herbal preparations on the Dutch market and to estimate the associated risks.

Summary

After an introduction to the topic of interest (Chapter 1) the thesis started with an overview of the present state-of-the-art in regulation and legislation of botanicals and botanical preparations used in food (Chapter 2). The need for adequate regulation and legislation is generally well recognized nowadays, especially given the rapid increase in consumer use, the over-the counter availability of botanicals and botanical preparations and the existence of serious safety concerns. In the Netherlands, regulation and legislation of botanicals and botanical preparations used in food covers naturally-occurring toxic compounds and contaminants present in these products, but it does not include provisions to counter the risk of interactions between certain botanical ingredients of foods and conventional drugs.

The Dutch Commodities Act Decree 'Herbal preparations' regulates the use of toxic plants and fungi in herbal preparations. It includes limits for three types of phytotoxins in herbal preparations, which are toxic pyrrolizidine alkaloids, aristolochic acids and yohimbe alkaloids. Furthermore, the Decree currently prohibits the use in herbal preparations of 46 plants and fungi that are too toxic to be used in food or in other commodities. The constituents responsible for the toxic effects of these 46 plants are also found in plants not specifically regulated by the Decree. Within Europe, EFSA developed a new methodology for the safety assessment of botanicals used in food supplements and published a Compendium of botanicals that raise toxicological concern to be used as input for risk assessments [8, 9]. European and Dutch risk assessment bodies concluded that several botanicals pose health risks. Chapter 2 presents an overview of the Dutch and European regulatory framework for selected food commodities with botanical ingredients as well as an overview of health concerns defined by risk assessors related to botanicals not specifically covered by these food safety laws. It is concluded that the current regulation and legislation in the Netherlands and Europe needs to be updated to address health risks relating to botanicals and botanical preparations that are identified to be of concern.

Naturally-occurring toxic compounds in botanicals

Given that one of the safety concerns over botanicals and botanical ingredients relates to natural toxins being present in these preparations, Chapter 3 presents an overview of the nature and mechanism of action of several toxic botanical ingredients that are, or will probably be, specifically covered by Dutch or European legislation and that are presently receiving increased attention in the field of food toxicology. This relates to compounds including aristolochic acids, pyrrolizidine alkaloids, beta-carotene, coumarin, the alkenylbenzenes safrole, methyleugenol and estragole, ephedrine alkaloids and synephrine, kavalactones, anisatin, St. John's wort ingredients, cyanogenic glycosides, solanine and chaconine, thujone, and glycyrrhizinic acid. It can be concluded that several of these phytotoxins cause concern, because of their bioactivation to reactive alkylating intermediates that are able to react with cellular macromolecules causing cellular toxicity, and, upon their reaction with DNA, genotoxicity resulting in tumors. Another group of the phytotoxins presented is active without the requirement for bioactivation and, in most cases, these compounds appear to act as neurotoxins interacting with one of the neurotransmitter systems. Altogether, the examples presented in Chapter 3 clearly illustrate that natural does not equal safe and that in modern society adverse health effects, upon either acute or chronic exposure to phytochemicals, can occur as a result of use of plant- or herb-based foods, teas, or other extracts.

Chapter 4 of this thesis subsequently reports data on the actual occurrence of a group of natural toxic botanical constituents of concern, namely aristolochic acids, in selected types of herbal preparations on the Dutch market. The occurrence of aristolochic acids I and II was monitored in traditional herbal preparations (THPs) used in Traditional Chinese Medicine (TCM). As discussed in Chapter 3, herbs with aristolochic acids and derivatives are nephrotoxic, genotoxic and carcinogenic. Several species from the family of the *Aristolochiaceae* that are used in TCM contain aristolochic acids. After aristolochic acid related nephropathy (AAN) and urothelial cancer were described in Belgium in female patients who had been exposed to aristolochic acids through contaminated herbal preparations, herbs with aristolochic acids were prohibited worldwide. Confusing nomenclature can result in contamination of certain Chinese THPs with aristolochic acids. The presence of aristolochic acids in THPs sampled on the Dutch market was analyzed using an LC-MS method. From 2002 up to and including 2006, 190 Chinese THPs were sampled using recent information on Chinese THPs potentially containing aristolochic acids. Aristolochic acid I was found in 25 samples up to a concentration of 1676 mg/kg. Aristolochic acid II was also found in 13 of these samples up to 444 mg/kg. All 25 positive samples including Mu Tong, Fang Ji, Tian Xian Teng and Xi Xin were part of a group of 68 THPs identified as possibly containing aristolochic acids. In a worst-case scenario, use of a sample of Mu Tong with the highest aristolochic acid content over a 7-day period would result in intake levels of aristolochic acids similar to the intake levels, which significantly raised the cancer risk in the Belgian AAN cases. Our results show that contaminated THPs still can be found on the market even after worldwide publicity. Therefore, it can be concluded that testing of possible aristolochic acid contamination of THPs is still essential.

EFSA noted that misidentification of plants harvested from the wild is a continuing problem causing the accidental use of toxic herbal species [2]. In addition to misidentification of plants in the wild, confusing nomenclature used for botanicals on the market can also result in misidentification and subsequent contamination

of food commodities with toxic plants of which we found evidence in our survey into aristolochic acids in Chinese THPs. From 2002 up to and including 2006 we sampled 14 preparations labeled as 'Mu Tong' of which relatively many (n=7) contained aristolochic acids (Chapter 4). The name Mu Tong refers to the stem of *Aristolochia manshuriensis* but it can also be used for the stem of 2 *Clematis* spp. and 2 *Akebia* spp. Of the 7 Mu Tong samples with aristolochic acids, 4 were indicated to contain exclusively the stem of *Akebia quinata* or *Akebia trifoliata.* Because *Akebia quinata* or *Akebia trifoliata* do not contain aristolochic acids, it was concluded that *Aristolochia* spp. were partially or completely substituting for these plants. Furthermore, one of these samples labeled to contain the stem of these two *Akebia* plants contained the highest amount of aristolochic acids found in the survey. This shows that misidentification or substitution of plants used in Chinese THPs is a risk that should be taken into account by producers or distributors of these products in the Netherlands.

The European Medicine Agency (EMA; previously the acronym EMEA was used) assessed in 2005 the evidence concerning Mu Tong and Fang Ji (the root of *Aristolochia fangchi* or 3 other herbs) substitutions and advised that in absence of appropriate quality control procedures the prohibition of species at risk of being confused with *Aristolochia* spp. should be considered. The plants posing a risk because of confusion with and substitution of *Aristolochia* spp. for these unrelated plants, include *Akebia quinata*, *Akebia trifoliata*, *Clematis armandii*, *Clematis montana*, *Cocculus orbiculatus*, *Cocculus laurifolius*, *Cocculus trilobus* and *Stephania tetrandra* [10]. Because *Stephania tetrandra* (Fang Ji or Han Fang Ji) is not toxic the Dutch Ministry of Health removed this botanical from the list of plants and fungi banned for use in herbal preparations in the Decree 'Herbal preparations' in 2005 [11]. RIVM and VWA advised however to maintain *Stephania tetrandra* on the list due to the potential for substitution with *Aristolochia* spp. [12, 13]. Currently, none of the herbs posing a risk because of confusion and substitution with *Aristolochia* spp. are banned in the Netherlands. Moreover, EMA advised to consider controlling other plant species of the *Aristolochiaceae* family, especially *Asarum* spp. that may contain aristolochic acids [10]. THPs with 'Xi Xin' (*Asarum sieboldii* herba) included in our study presented in Chapter 4, frequently contained aristolochic acids. Of 12 products with Xi Xin, 11 contained relatively low levels of aristolochic acid I. Possible implications of these findings are discussed further on in this Chapter in the section on future perspectives.

Contamination of herbal preparations

In addition to toxicological concerns over naturally occurring toxic compounds in botanicals, the safety of food commodities with botanical ingredients can also become compromised through contamination, intentionally or by accident, with a multitude of substances, including, for example, polycyclic aromatic hydrocarbons (PAH) or metals such as lead. In traditional medicine especially botanicals are frequently used, although animal parts and/or minerals are also applied [14]. In several Asian traditional medicine systems such as Ayurveda, Unani-Tibb, Siddha, TCM and Traditional Tibetan Medicine (TTM), ingredients that contain significant amounts of mercury, arsenic or lead are deliberately added to certain herbal preparations for therapeutic purposes. Although these ingredients are widely considered to be safe in these traditional medicine systems, they have caused intoxications worldwide. The aim of the study presented in Chapter 5 of this thesis was therefore to determine

mercury, arsenic and lead levels in Asian THPs on the Dutch market. 292 THPs used in Ayurveda, TCM and TTM were sampled between 2004 and 2007. Samples were mostly multi ingredient THPs containing herbs and minerals. The labeling of less than 20% of the THPs suggested the presence of mercury, arsenic or lead. These elements were shown by ICP-MS to be present in 186 (64%) of 292 THPs. Estimated weekly mercury, arsenic and lead intake levels were calculated for each THP from the analytically determined concentrations and the recommended dose. 59 THPs (20%) were likely to result in intakes of these elements significantly exceeding safety limits. Of these 59 THPs, intake estimates for 50 THPs significantly exceeded the safety limit for mercury (range 1.4-1747 mg/week); intake estimates for 26 THPs significantly exceeded the safety limit for arsenic (range 0.53-427 mg/week) and intake estimates for 8 THPs were significantly above the safety limit for lead (range 2.6-192 mg/week). It was concluded that the mercury, arsenic and lead contents of THPs used in Ayurveda, TCM and TTM remain a cause for concern and require strict control.

The survey into lead, mercury and arsenic in Asian THPs presented in Chapter 5 was continued in 2008. The results of the 2008 survey do not indicate that the safety of these products has improved significantly that year compared to the results obtained in the previous years. We found in 2008, mercury, lead and arsenic in 60 (77%) of 78 Asian THPs. When used at the recommended dose level, four of these THPs would result in arsenic or mercury intakes above the safety limits selected in Chapter 5. Mercury levels of 27 THPs exceeded the recently established EU limit for mercury in food supplements of 0.1 mg/kg on a total of 28 THPs in which mercury was found. Lead levels of 36 THPs were above the new EU limit for lead in food supplements of 3.0 mg/kg on a total of 56 THPs in which lead was found [15].

Recently, EFSA assessed the risks of lead and arsenic intakes from food and from these risk assessments it can be concluded that current levels of exposure to lead and arsenic from food are of more concern than previously considered. This also affects the risks linked to Asian THPs with lead or arsenic as estimated in Chapter 5. The EFSA Panel on Contaminants in the Food Chain (CONTAM) concluded in its Scientific Opinion on Arsenic in Food of October 2009 that the provisional tolerable weekly intake (PTWI) of 15 µg/kg bw established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is no longer appropriate. The CONTAM Panel identified a range of values for the 95% lower confidence limit of the benchmark dose of 1% extra risk (BMDL01), for use in the risk characterization for inorganic arsenic. The BMDL01 values for the relevant health endpoints, i.e. skin lesions, cancers of the skin, urinary bladder and lung, ranged from 0.3 to 8 µg/kg bw/day [16]. In our study on arsenic, lead and mercury in Asian THPs sampled before 2008 presented in Chapter 5, we used a TDI of 1.0 µg/kg bw/day established by RIVM. This TDI was based on the JECFA PTWI to which an extra uncertainty factor of 2 was applied for observation errors in epidemiological studies [17]. The TDI established by RIVM for inorganic arsenic lies at the lower end of the range of BMDL01 values defined by the CONTAM Panel. In our study presented in Chapter 5, 26 of 292 Asian THPs analyzed showed arsenic levels that when used at recommended dose levels, would result in intakes significantly above the RIVM TDI. Furthermore, another 77 of the total also contained arsenic but at levels that would result in exposure levels that do not exceed the TDI of 1.0 µg/kg bw/day significantly when used according recommendations on the label. For the majority of samples in this group a margin of safety of one or two orders of magnitude exists between estimated arsenic

intake levels for these samples at the recommended dose levels and the RIVM TDI. Since the TDI lies in the range of BMDL01 values identified by the CONTAM Panel, this margin can also be considered representative for the range of BMDL01 values identified by the CONTAM Panel.

Because no maximum level for arsenic applies to food supplements in European or Dutch food law, the VWA has to proof that a supplement with relatively high arsenic levels is injurious to health or unfit for human consumption before management actions can be taken. The placing on the market of products, which are injurious to health or unfit for human consumption, is prohibited by the General Food Law (European Regulation (EC) No 178/2002) which provisions directly apply in the Netherlands [18]. In view of the CONTAM Panel Opinion on Arsenic in Food it should be considered whether it is still appropriate to use the RIVM TDI for inorganic arsenic to assess if a food supplement with a relatively high arsenic level can be used safely. The setting of maximum limits for arsenic in food supplements would help the enforcement of the safety of these products and should therefore be considered.

Furthermore, the CONTAM Panel also noted that food supplements based on algae were among the food commodities in which the highest total arsenic levels were measured. This is especially relevant for several algae such as hijiki (*Hizikia fusiforme*) that can contain high total arsenic levels of which up to 60% consists of inorganic arsenic besides organic arsenic compounds that are of lower toxicological concern [16, 19]. In other algae inorganic arsenic makes up only a small percentage of total arsenic levels [19]. The CONTAM Panel also stressed the need for data on arsenic speciation in different food commodities in order to refine risk assessments [16].

The CONTAM Panel published in April 2010 an Opinion on the risk assessment for lead in food. The Panel concluded in its Scientific Opinion on lead in food that the PTWI of 25 µg/kg bw established by JECFA is not appropriate anymore. Because there was no evidence for a threshold for a number of critical endpoints including developmental neurotoxicity and nephrotoxicity in adults, the Panel considered it to be not appropriate to derive a PTWI. It was concluded that there is potential concern at current levels of exposure to lead for effects on neurodevelopment in infants, children and pregnant women. Measures taken to protect children and women of child-bearing age against the potential risk of neurodevelopmental effects will also protect against all other adverse effects of lead, in the population as a whole. The Panel derived a BMDL01 intake level of 0.50 µg/kg bw per day for neurodevelopmental effects [20]. In our study on arsenic, lead and mercury in Asian THPs (Chapter 5) we used a TDI of 3.6 µg/kg bw/day established by RIVM which was directly based on the JECFA PTWI for lead [17]. Because EFSA does no longer consider the JECFA PTWI for lead to be appropriate, the use by the VWA of the corresponding TDI adopted by RIVM for food commodities to which no legal maximum limits applies, should also be reconsidered.

The mercury detected in Ayurvedic THPs, as reported in Chapter 5, may result from the fact that in Ayurveda, mercury is deliberately added to several THPs in the form of preparations called ‘bhasmas’. Metals or metalloids in bhasmas are claimed to be detoxified by rigorous processing. The toxicity of mercury depends on the chemical form in which it exists [21, 22]. Mercury in its elemental state, referred to as Hg(0), shows a low oral toxicity while inorganic and organic mercury forms are significantly more toxic [22, 23]. As shown in Chapter 5 we

determined mercury in 242 Ayurvedic THPs and use of 19% of these preparations at the recommended dose level would result in mercury intakes above a toxicological safety limit for inorganic mercury. The study presented in Chapter 6 is aimed at investigating whether (part of) the mercury detected in these Ayurvedic THPs is present as Hg(0), which would provide an argument to reconsider the safety assessment using limits for inorganic mercury. To test this we developed a method that enabled detection of Hg(0) in these Ayurvedic THPs and subsequently performed a study on the presence of Hg(0) in 19 Ayurvedic THPs of which the study in Chapter 5 showed that mercury intakes with the recommended daily dose exceeded the safety limit for inorganic mercury. The results obtained reveal that in 11 of the 19 THPs analyzed all mercury detected could be accounted for by other forms than Hg(0). Extraction of the other 8 THPs with 7M HNO_3, a method demonstrated to efficiently extract Hg(0), revealed that most of the mercury present in these samples (i.e. 69-99%) could not be extracted by 7M HNO_3, pointing at other forms than Hg(0) being present. It was concluded that in all THPs the main part of the mercury content is not present in its relatively non-toxic elemental form, that the mercury detected in Ayurvedic THPs is likely to be present in the inorganic form and that therefore the estimation of the related risks based on the safety limits for inorganic mercury would be justified. Finally, no additional risk assessments of inorganic mercury by national or international expert committees have been identified to date.

The metals and metalloids encountered in Asian THPs are most likely the result of intentional addition and differ in that respect from contaminants such as polycyclic aromatic hydrocarbons (PAH) that can occur in food through several processes. PAH are also found in food supplements and the Dutch Commodities Act includes since January 2006 a maximum level of 10 µg/kg benzo[a]pyrene for botanical food supplements and a limit of 2 µg/kg for supplements without botanical ingredients [24]. EFSA has defined 16 priority PAH that are both genotoxic and carcinogenic and identified 8 priority PAH (PAH8) or 4 of these (PAH4) as good indicators of the toxicity and occurrence of PAH in food. The study described in Chapter 7 aims to determine benzo[a]pyrene and other EFSA priority PAH in different categories of food supplements containing botanicals and other ingredients. In 2003 up to 2008, benzo[a]pyrene exceeded the limit of quantification (LOQ) in 553 (44%) of 1258 supplements with a lower bound mean of 3.37 µg/kg. In 2008 and 2009, benzo[a]pyrene and 12 other EFSA priority PAH were determined in 333 food supplements. Benzo[a]pyrene exceeded the LOQ in 210 (63%) food supplements with a lower bound mean of 5.26 µg/kg. Lower bound mean levels for PAH4 and PAH8 (-indeno[1,2,3-cd]pyrene) were 33.5 µg/kg and 40.5 µg/kg, respectively. Supplements containing resveratrol, *Ginkgo biloba*, St. John's wort and propolis showed relatively high PAH levels in 2008 and 2009. Before 2008, supplements with these ingredients and also dong quai, green tea or valerian contained relatively high benzo[a]pyrene levels. On average, PAH intake resulting from food supplement use will be at the lower end of the range of contributions of main food groups to PAH exposure, although individual food supplements can contribute significantly to PAH exposure. Regular control of PAH levels in food supplements may prove a way forward to further reduce the intake of PAH from food. It was interesting to note that overall in 192 (58%) of the supplements in the 2008 and 2009 survey (n=333), PAH4 levels were lower than 10 µg/kg and in 99 (30% of the total) of these PAH4 levels were below the LOQ. Furthermore, in all subcategories of food supplements identified in this survey

there were supplements that contained no quantifiable or relatively low levels of PAH. This suggests that contamination of food supplements with PAHs is avoidable.

Overall, on the basis of the surveys on contamination of herbal preparations on the Dutch market with aristolochic acids, lead, mercury, arsenic and PAH of which the analytical data are presented in this PhD thesis it can be concluded that contamination of herbal preparations with PAH is most widespread, followed by contamination with mercury, arsenic or lead, and with aristolochic acids in the last place. The toxicity of these different substances cannot easily be compared. When however for each of these contaminants the effects of exposure at the high end of the range of estimated intake levels identified in this thesis during a certain amount of time would be compared, the effects found for aristolochic acids would likely be most detrimental to health, followed by the toxic effects of lead, arsenic, mercury and PAH, in that particular order. However, the facts that aristolochic acids were only found in a small segment of the products sold in TCM outlets in the Netherlands, and that considerably more Asian THPs were found on the market that contained lead, arsenic or mercury, would suggest that Asian THPs with relatively high levels of lead, arsenic or mercury are currently more likely to cause adverse effects in the Netherlands. Estimated exposure levels to PAH from food supplements identified in Chapter 7 are within the range of exposure levels to PAH from food identified in the European Union and the associated health effects are therefore of a lower order than the effects of aristolochic acids, lead, mercury and arsenic. However, because several PAH are genotoxic and carcinogenic and exposure to PAH from food is of potential concern for high level consumers, efforts should be made to reduce exposure to PAH from food supplements to further decrease the exposure from food.

Future perspectives

The European Commission concluded in 2008 that laying down specific European rules for substances other than vitamins and minerals for use in food supplements is not justified [25]. This means that it is not likely that in the foreseeable future a European counterpart to the Dutch Commodities Act Decree 'Herbal preparations' will be established. Regulation (EC) No 1925/2006 establishes a procedure in article 8 to be used in cases where a substance other than vitamins or minerals (e.g. a botanical) might represent a potential risk to consumers. The Commission may take, based on an EFSA safety assessment or information provided by the Member States, the decision to prohibit or restrict the use of this substance in food. The safety of botanical ingredients can be assessed using the EFSA approach for the safety assessment of botanicals and botanical preparations in combination with the EFSA Compendium of botanicals reported to contain toxic, addictive, psychotropic, or other substances of concern. No decisions have yet been taken using this procedure. It is yet not clear whether the Commission will make much use of the procedure in article 8 of Regulation (EC) No 1925/2006 to restrict or prohibit certain botanicals for use in foods.

EFSA already pointed out in 2004 that the use of botanicals and botanical preparations in food is increasing [2]. Safety concerns related to botanicals will therefore increase in significance as well. Because new European legislation for botanicals and botanical preparations intended to be used in food supplements is not

likely to be established in the near future, it would be prudent to keep legislation such as the Decree 'Herbal preparations' up to date in order to protect Dutch consumers from serious risks resulting from use of herbal preparations. When an update of the Decree 'Herbal preparations' is initiated, this update should take into account risk assessments and recommendations on botanical products by national and European risk assessment agencies such as RIVM, EMA and EFSA.

Several risk assessment bodies have advocated label warnings for a number of botanicals. For example, EMA defined warnings related to interactions of herbal medicinal products with St. John's wort with other medicinal products [26]. Because St. John's wort is also used in botanical food supplements and other herbal preparations, these warnings might be of significance for all St. John's wort-containing products for oral use on the market. In addition, a number of risk assessors recommended that preparations with black cohosh (*Actaea racemosa* or *Cimicifuga racemosa)* should carry warnings regarding the potential of this herb to cause liver dysfunction [27]. It should be considered to include in the Decree 'Herbal preparations' compulsory label warnings for certain herbal preparations for which health concerns exist, but which could be used safely when consumers are informed how specific adverse health effects from this product can be avoided. However, it would then be advisable to establish for which types of adverse effects this approach is appropriate. Although it is not customary in the Netherlands for foods to carry label warnings, compulsory label warnings have been established for foods with certain levels of glycyrrhizinic acid. Currently, the rule does not apply to herbal preparations such as botanical food supplements. However, it should be considered to extend the label warning to herbal preparations in which *Glycyrrhiza* species are used that contain glycyrrhizinic acid.

Furthermore, it is important to note that the introduction of label warnings on a product that is linked to certain adverse health effects does not guarantee that these health problems will cease to occur. This is illustrated by differences in approaches worldwide to protect consumers against kava kava toxicity. Use of this botanical is linked to liver problems and kava kava was banned in herbal preparations for this reason in the Netherlands in 2003. In the USA, kava kava remained on the market. The US herbal industry incorporated warnings about possible liver problems into their labeling and the US Food and Drug Administration (FDA) issued warnings as well. Despite these measures, reports of hepatotoxicity continued to be received by the FDA. Australia also introduced label warnings about rare cases of liver problems upon intake of kava kava but reports of hepatotoxicity continued to be received there as well [28].

EFSA concluded in 2004 that the growing volume of sales in the European Union with products obtained from suppliers based in Asia or elsewhere and the move towards widespread outlets for the products of traditional medicine call for more formal pre-marketing assessment and more stringent controls than the occasional random checks and analyses often carried out by individual national or local authorities on what is already out in the market [2]. At present the Dutch or European regulatory framework for food commodities with botanical ingredients presented in Chapter 2 does not include provisions that enable stringent pre-marketing assessments for all products that are brought on the market. It is however possible to increase the frequency of surveys into naturally-occurring toxic constituents of botanicals and contaminants in food supplements that are already on the market. This approach can result in improvements of the safety of these products, although this may prove to be

a lengthy process. For instance, there are signs that enforcement actions by the VWA have caused a decrease in the number of THPs with aristolochic acids on offer in retail channels. In 2008, the VWA sampled 77 Chinese THPs for analysis of aristolochic acids and in one THP traces of aristolochic acids were detected [15]. In contrast, aristolochic acids were found in 25 of 190 Chinese THPs that were sampled from 2002 up to and including 2006 (Chapter 4 of this thesis). But conclusions on a potential reduction in the risk of contamination of herbal preparations with aristolochic acids in Dutch retail channels should be based on results of several more years of market surveys. On the basis of future results it can then be assessed whether *Asarum* species and the plants at risk of substitution with *Aristolochia* species should be prohibited from use in herbal preparations. Another reason to assess the safety of the use of *Asarum* species in herbal preparations on the market is the possible presence of alpha-asarone, which is a mammalian carcinogen [29]. It should also be noted that consumers might come into contact with products containing aristolochic acids via channels such as Internet, which complicates official controls. Additionally, it is imperative that food business operators who bring THPs on the market, which in theory could contain aristolochic acids either directly because of the intended presence of plants from the *Aristolochiaceae* family, or unintentionally through confusion of intended botanical ingredients with *Aristolochia* spp., include measures in their quality control (Hazard Analysis Critical Control Points - HACCP) procedures in order to prevent this hazard.

The CONTAM Panel Opinions on arsenic and lead [16, 20] are of significance for the enforcement of the safety of herbal preparations and other food supplements. It should be considered if it is still appropriate to use the RIVM TDI for inorganic arsenic and the RIVM TDI for lead to assess whether a food supplement with a relatively high arsenic or a relatively high lead level can be used safely. The setting of maximum levels for arsenic in food supplements would help the enforcement of the safety of these products and should therefore be considered. Also data on arsenic and mercury speciation in different food commodities is needed in order to refine risk assessments [16].

Regarding the future setting of maximum levels for PAH in food supplements in European food safety law it should be noted that in our survey on PAH in food supplements presented in Chapter 7 in nearly 60% of the supplements in the 2008 and 2009 survey PAH4 levels were lower than 10 µg/kg. Furthermore, there were supplements present in all subcategories of food supplements that contained no quantifiable or relatively low levels of PAH present. This suggests that contamination of food supplements with PAHs is avoidable. These findings may be of significance for the setting of European maximum levels for PAH in food supplements, which may prove a way forward to further reduce the intake of PAH from food.

Conclusions

From the work described in this thesis it can be concluded that for herbal preparations ‘natural’ does not equal ‘safe’. Especially the results presented in Chapters 4 and 5 show that the mere fact that a herbal preparation has been brought on the Dutch market is not a guarantee that the product is safe and that it may not cause adverse health effects. Work done by national and European risk assessment agencies such as RIVM,

EMA and EFSA and reports in literature as discussed in Chapters 2 and 3 shows that certain herbal preparations other than those that are at present specifically covered by Dutch food safety law, raise toxicological concern as well. Furthermore, consumers should be aware that the risk of adverse health effects increases when herbal preparations are purchased outside regular retail channels, as is the case when preparations are acquired via Internet, traditional medicine outlets, smartshops or similar types of outlets. While food inspection authorities direct considerable effort to enforce food safety in the food supplement field, the primary legal responsibility for the safety of the food products is on the business operators who place these products on the market. It is imperative that food business operators who bring herbal preparations on the market acquire in-depth information on the hazards relevant to these products and include measures in their quality control (HACCP) procedures that aim to prevent, eliminate or reduce all significant hazards to acceptable levels. The fact that a food inspection authority has not yet analyzed products from a food business operator for the presence of certain constituents that raise toxicological concern or examined whether the operator has implemented measures aimed at preventing, eliminating or reducing these hazards to acceptable levels, does not mean that these hazards do not require action by the food operator. Finally, the European Commission concluded that new European legislation for botanicals and botanical preparations intended to be used in food supplements is not necessary and that existing legislation could be used to restrict or prohibit the use in food of certain toxic substances such as certain botanicals. Because it is yet not clear whether the European Commission will make much use of these possibilities it would be prudent to keep the Dutch Decree 'Herbal preparations' and other national legislation up to date in order to protect consumers from serious risks resulting from use of botanicals in food products such as herbal preparations.

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Samenvatting en discussie
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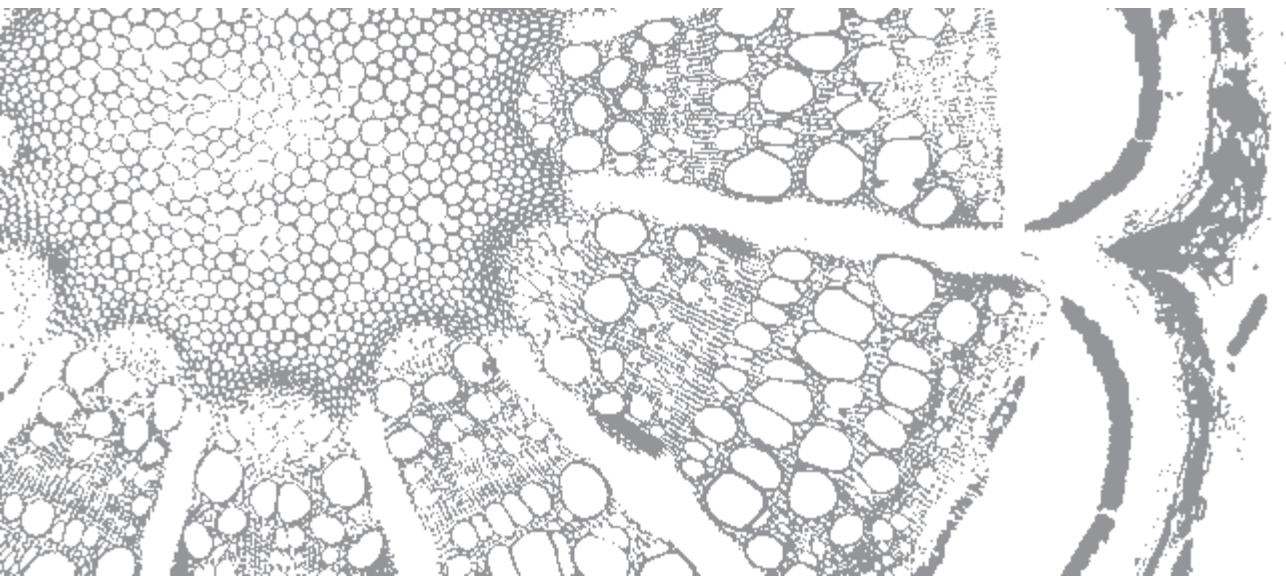

SAMENVATTING EN DISCUSSIE

Inleiding

Gedurende de laatste twintig jaar is het in toenemende mate duidelijk geworden dat de beschikbaarheid en het gebruik van kruidenpreparaten buiten het geneesmiddelencircuit in Nederland en in andere Lidstaten van de Europese Unie aan het groeien was. Er kwam in deze periode ook meer aandacht voor kruiden op de markt die acuut toxisch zijn of die een schadelijke werking hebben op de lange termijn [1, 2]. In 2004 onderkende tevens de Europese Autoriteit voor voedselveiligheid (the European Food Safety Authority - EFSA) dat met de groeiende populariteit van voedingssupplementen die kruidenpreparaten bevatten eveneens de gevaren toenamen. De gevaarseigenschappen van kruiden en kruidenpreparaten houden verband met i) het voorkomen van natuurlijke toxinen in planten, ii) de opzettelijke toevoeging of toevallige aanwezigheid van toxische contaminanten of iii) interacties van kruideningrediënten met werkzame ingrediënten uit geneesmiddelen [2].

De aanwezigheid van toxische stoffen in kruidenpreparaten die van nature voorkomen in de planten die in de preparaten verwerkt zijn, is een belangrijke reden tot zorg. Het Warenwetbesluit Kruidenpreparaten stelt daarom regels aan de aanwezigheid van specifieke toxische stoffen zoals aristolochiazuren in kruidenpreparaten. Dit 'Kruidenbesluit' trad in werking in 2001 en is van toepassing op het gebruik van planten en schimmels in levensmiddelen en andere waren. Kruidenpreparaten worden door het Kruidenbesluit gedefinieerd als kruidensubstanties, al dan niet bewerkt, die bestemd zijn om te worden gebruikt door de mens, daaronder begrepen kruidenextracten. Daarbij zijn kruidensubstanties gedefinieerd als substanties bestaande uit plantenmateriaal. In aanvulling op regels die de aanwezigheid van meerdere giftige stoffen in kruidenpreparaten beperken, verbiedt het besluit tevens het gebruik van bepaalde giftige kruiden en schimmels in kruidenpreparaten [1]. De begrippen 'kruidensubstanties' en 'kruidenpreparaten' zijn buiten de wettelijke context min of meer synoniem aan respectievelijk de begrippen 'botanicals' en 'botanical preparations' die door EFSA worden gebruikt.

Een tweede reden voor bezorgdheid over de veiligheid van kruidenpreparaten is de opzettelijke toevoeging of onbedoelde aanwezigheid van een reeks aan contaminanten. Vervuiling met lood, arseen en kwik is beschreven voor kruidenpreparaten uit verschillende Aziatische traditionele geneeswijzen en gebruik van dergelijke vervuilde kruidenpreparaten heeft wereldwijd tot vergiftigingen geleid [3]. In de traditionele Indiase geneeswijze Ayurveda, de traditionele Tibetaanse geneeswijze en in de traditionele Chinese geneeswijze worden voor therapeutische doeleinden de metalen kwik en lood en het metalloïde arseen opzettelijk toegevoegd aan kruidenpreparaten. Metalen en metalloïden zoals kwik en arseen kunnen voorkomen in verschillende chemische vormen die in toxiciteit aanmerkelijk van elkaar kunnen verschillen. Voor het schatten van de risico's van metalen en metalloïden in voeding is informatie over de chemische vorm waarin deze stoffen aanwezig zijn van belang. Andere contaminanten die in kruidenpreparaten gevonden kunnen worden zijn polycyclische aromatische koolwaterstoffen (PAK's). Vervuiling met PAK's kan het resultaat zijn van het toepassen op levensmiddelen van daarvoor ongeschikte productieprocessen zoals het direct drogen van plantenmateriaal met verbrandingsgassen. Contaminatie kan ook plaatsvinden doordat PAK's uit het milieu neerslaan op gewassen. In bepaalde

productieprocessen toegepaste extractiestappen kunnen deze PAK's vervolgens concentreren wat in het verleden heeft geleid tot contaminatie van natuurlijk vitamine E met hoge concentraties aan PAK's [4-6]. Meerdere PAK's zoals benzo[a]pyreen zijn genotoxisch en carcinogeen. Verschillende expertcomités hebben risicobeoordelingen van PAK's uitgevoerd. Een panel van EFSA heeft 16 PAK's aangewezen die prioriteit moeten krijgen in het onderzoek. EFSA concludeerde dat 8 van deze 16 prioriteits-PAK's waarvoor gegevens over carcinogeniteit beschikbaar zijn (de PAK8) gebruikt kunnen worden als indicator van de carcinogene eigenschappen van PAK's in de voeding. De meest geschikte indicator voor PAK's voor wat betreft de toxiciteit en het voorkomen in voeding, is op dit moment een combinatie van 4 van de individuele verbindingen uit de PAK8 (de PAK4) of de PAK8 zelf [7].

Tenslotte geeft het optreden van interacties tussen bepaalde kruiden en conventionele geneesmiddelen aanleiding tot zorg. Deze interacties kunnen de gezondheid ernstig schaden. Sint-janskruid is een bekend voorbeeld van een kruid waarvan het gelijktijdig gebruik met bepaalde geneesmiddelen kan leiden tot ernstige interacties. Het mechanisme achter de interacties tussen sint-janskruid en deze geneesmiddelen wordt in Hoofdstuk 3 van dit proefschrift besproken. Interacties tussen andere kruiden en geneesmiddelen worden verder niet behandeld in dit proefschrift.

Het doel van dit proefschrift was het beschrijven van de toxiciteit van bepaalde kruidenpreparaten, het vaststellen van de aanwezigheid en concentraties van bepaalde contaminanten en natuurlijke toxinen in kruidenpreparaten op de Nederlandse markt en het schatten van de geassocieerde risico's.

Samenvatting

Nadat in Hoofdstuk 1 het onderzoeksonderwerp is geïntroduceerd, wordt in Hoofdstuk 2 een overzicht gegeven van de huidige wet- en regelgeving voor kruidenpreparaten die als levensmiddel toegepast worden. Door de snelle toename in het gebruik van kruidenpreparaten, de brede beschikbaarheid van deze producten en de kans op ernstige bijwerkingen wordt de noodzaak tot adequate wet- en regelgeving voor deze producten tegenwoordig breed erkend. In Nederland zijn in de levensmiddelenwetgeving normen opgenomen voor natuurlijke toxinen en contaminanten in kruidenpreparaten maar er ontbreken in deze wetgeving bepalingen die gericht zijn op het verminderen van de kans op interacties tussen bepaalde kruidenpreparaten en reguliere geneesmiddelen. Het Nederlandse Warenwetwetbesluit Kruidenpreparaten (ook wel het Kruidenbesluit) reguleert het gebruik van giftige planten en schimmels in kruidenpreparaten. Het Kruidenbesluit bevat normen voor drie plantentoxinen in kruidenpreparaten: toxische pyrrolizidine alkaloïden, aristolochiazuren en yohimbe alkaloïden. Daarnaast verbiedt het Kruidenbesluit op dit moment het gebruik in kruidenpreparaten van 46 planten en schimmels die te giftig zijn om in levensmiddelen of in bepaalde andere waren te gebruiken. De verbindingen die verantwoordelijk zijn voor de giftige effecten van deze 46 planten en schimmels worden ook gevonden in andere planten of schimmels die niet in het Kruidenbesluit zijn opgenomen. Op Europees niveau ontwikkelde EFSA een nieuwe methodologie voor de veiligheidsbeoordeling van kruidenpreparaten die in voedingssupplementen gebruikt worden. EFSA publiceerde tevens een Compendium van kruiden waarvan de veiligheid ter discussie

staat. Informatie uit het Compendium kan vervolgens als beginpunt voor risicoschattingen dienen [8, 9]. Europese en Nederlandse risicobeoordelaars concludeerden dat verschillende kruidenpreparaten een risico voor de gezondheid kunnen zijn. Hoofdstuk 2 geeft een overzicht van de Nederlandse en Europese wettelijke bepalingen voor levensmiddelen met kruidenbestanddelen. Tevens worden in dit hoofdstuk risicoschattingen besproken van meerdere kruiden waarvoor geen specifieke wettelijke maatregelen zijn genomen. Geconcludeerd kan worden dat de huidige wettelijke bepalingen in Nederland en Europa moeten worden aangepast om te voorkomen dat kruidenpreparaten die aanleiding tot zorg geven de gezondheid van gebruikers kunnen schaden.

Natuurlijke toxinen in kruiden

Omdat de aanwezigheid van natuurlijke toxinen in kruidenpreparaten een belangrijke reden tot zorg is, richt Hoofdstuk 3 zich op het werkingsmechanisme en de aard van verschillende toxische kruidensubstanties die op dit moment extra in de belangstelling staan in de levensmiddelentoxicologie en waarvoor specifieke maatregelen in de Nederlandse of Europese wetgeving genomen zijn of - waarschijnlijk - genomen zullen worden. Dit betreft kruidenbestanddelen zoals aristolochiazuren, pyrrolizidine alkaloïden, bèta-caroteen, coumarine, de alkenylbenzeensubstanties safrol, methyleugenol en estragol, efedrine alkaloïden en synefrine, kavalactonen, anisatine, sint-janskruidbestanddelen, cyanogene glycosiden, solanine en chaconine, thujon en glycyrrhizinezuur. Geconcludeerd kan worden dat meerdere van deze fytotoxinen aanleiding tot zorg geven door bioactivatie tot reactieve alkylerende metabolieten die kunnen reageren met cellulaire macromoleculen leidend tot cellulaire toxiciteit en, in geval van een reactie met DNA, genotoxiciteit en tumoren. Een andere groep van de besproken fytotoxinen is toxisch zonder bioactivatie en deze stoffen zijn in de meeste gevallen neurotoxinen die op neurotransmittersystemen inwerken. Alles samengenomen illustreren de in Hoofdstuk 3 besproken voorbeelden duidelijk dat het begrip ‘natuurlijk’ niet gelijk staat aan ‘veilig’ en dat in de huidige maatschappij schadelijke gezondheidseffecten kunnen optreden door acute of chronische blootstelling aan fytotoxinen als gevolg van het gebruik van levensmiddelen op basis van kruiden of ander plantenmateriaal, kruidenthees of andere kruidenextracten.

Hoofdstuk 4 van dit proefschrift beschrijft vervolgens de resultaten van onderzoek naar het daadwerkelijke voorkomen van een groep van natuurlijke toxische bestanddelen van kruiden die vanuit toxicologisch oogpunt reden tot zorg vormen. In het hoofdstuk wordt het voorkomen onderzocht van aristolochiazuren I en II in specifieke typen kruidenpreparaten op de Nederlandse markt, namelijk traditionele kruidenpreparaten (TKP’s) die worden toegepast in de Traditionele Chinese Geneeswijze (TCG). Zoals reeds besproken in Hoofdstuk 3 zijn kruiden met aristolochiazuren en derivaten daarvan nefrotoxisch, genotoxisch en carcinogeen. Meerdere soorten uit de familie van de *Aristolochiaceae* die gebruikt worden in TCG bevatten aristolochiazuren. Nadat aristolochiazuurgerelateerde nierschade (aristolochic acid related nephropathy - AAN) en kanker aan de urinewegen beschreven was in België bij vrouwelijke patiënten die waren blootgesteld aan aristolochiazuren uit gecontamineerde kruidenpreparaten, werden kruiden met aristolochiazuren wereldwijd verboden. Verwarrende nomenclatuur kan tot gevolg hebben dat bepaalde Chinese TKP’s verontreinigd raken met aristolochiazuren. De aanwezigheid van aristolochiazuren in TKP’s op de Nederlandse markt werd

geanalyseerd met een LC-MS methode. Vanaf 2002 tot en met 2006 werden 190 Chinese TKP's bemonsterd op basis van recente informatie over Chinese TKP's die mogelijk aristolochiazuren bevatten. Aristolochiazuur I werd in 25 monsters gevonden tot een concentratie van 1676 mg/kg. Tevens werd aristolochiazuur II in 13 van deze monsters gevonden tot een concentratie van 444 mg/kg. Alle 25 positieve monsters waaronder Mu Tong, Fang Ji, Tian Xian Teng en Xi Xin maakten deel uit van een groep van 68 THPs die geïdentificeerd waren als mogelijk gecontamineerd met aristolochiazuren. In een worstcase scenario zou het gebruik van het monster Mu Tong met het hoogste aristolochiazuurgehalte in de studie gedurende een periode van 7 dagen resulteren in een blootstelling aan aristolochiazuren vergelijkbaar met de blootstellingsniveaus die in de Belgische AAN-casussen geassocieerd waren met een verhoogd kankerrisico. Onze resultaten tonen aan dat zelfs na wereldwijde publiciteit gecontamineerde TKP's nog steeds op de markt gevonden kunnen worden. Het kan daarom geconcludeerd worden dat het testen op mogelijke contaminatie van TKP's met aristolochiazuren nog steeds noodzakelijk is.

EFSA is van mening dat misidentificatie van in het wild geoogste planten een blijvend probleem is dat kan leiden tot het onbedoelde gebruik van toxische plantensoorten [2]. In aanvulling op misidentificatie in het wild kan het gebruik van verwarrende nomenclatuur voor kruidenpreparaten op de markt leiden tot misidentificatie en daarop volgend tot contaminatie van levensmiddelen met toxische planten. Een voorbeeld hiervan vonden we in ons onderzoek naar aristolochiazuren in Chinese TKP's. Vanaf 2002 tot en met 2006 bemonsterden we 14 preparaten met de aanduiding 'Mu Tong' waarvan relatief veel (n=7) aristolochiazuren bevatten (Hoofdstuk 4). De naam Mu Tong duidt op de stengel van *Aristolochia manshuriensis* maar het kan ook gebruikt worden voor de stengel van 2 *Clematis* spp. en 2 *Akebia* spp. Van de 7 Mu Tong monsters met aristolochiazuren waren 4 zo aangeduid dat deze preparaten alleen de stengel van *Akebia quinata* of *Akebia trifoliata* zouden bevatten. Omdat *Akebia quinata* en *Akebia trifoliata* geen aristolochiazuren bevatten, werd geconcludeerd dat deze preparaten gedeeltelijk of volledig door *Aristolochia* spp. gesubstitueerd waren. Daarbij werd in één van de monsters waarvan uit de aanduiding bleek dat alleen de stengel van deze twee soorten *Akebia* planten aanwezig zou zijn, de hoogste hoeveelheid aan aristolochiazuren uit het onderzoek gevonden. Dit toont aan dat misidentificatie of substitutie van kruiden in Chinese TKP's een risico is waarmee rekening gehouden moet worden door producenten en distributeurs van dergelijke producten in Nederland.

De Europese Geneesmiddelen Autoriteit (European Medicine Agency - EMA; eerder werd de afkorting EMEA gebruikt) onderzocht in 2005 het bewijs voor substitutie van Mu Tong en Fang Ji (de wortel van *Aristolochia fangchi* of van 3 andere kruiden) en adviseerde daarbij dat in de afwezigheid van geschikte kwaliteitscontroleprocedures een verbod op de plantensoorten die verward zouden kunnen worden met *Aristolochia* spp. zou moeten worden overwogen. De niet aan *Aristolochia* spp. verwante planten die bewust of onbewust verwisseld kunnen worden met *Aristolochia* spp. en zo een risico vormen zijn *Akebia quinata*, *Akebia trifoliata*, *Clematis armandii*, *Clematis montana*, *Cocculus orbiculatus*, *Cocculus laurifolius*, *Cocculus trilobus* en *Stephania tetrandra* [10]. Omdat *Stephania tetrandra* (Fang Ji of Han Fang Ji) niet toxisch is, verwijderde het Nederlandse Ministerie van Volksgezondheid in 2005 dit kruid van de lijst uit het Warenwetbesluit Kruidenpreparaten met planten en schimmels die niet in kruidenpreparaten gebruikt mogen worden [11]. Het

RIVM en de VWA adviseerden echter om *Stephania tetrandra* op de lijst te handhaven vanwege de kans op substitutie met *Aristolochia* spp. [12, 13]. Op dit moment is geen van de kruiden die een risico vormen voor verwarring en substitutie met *Aristolochia* spp. in Nederland verboden. EMA adviseerde eveneens om andere soorten uit de familie van de A*ristolochiaceae* te reguleren, in het bijzonder *Asarum* spp. die ook aristolochiazuren kunnen bevatten [10]. TKP's met 'Xi Xin' (*Asarum sieboldii* herba) in ons onderzoek dat in Hoofdstuk 4 is gepresenteerd, bevatten in meerdere gevallen aristolochiazuren. Van de 12 producten met Xi Xin bevatten 11 relatief lage concentraties aristolochiazuur I. Mogelijke implicaties van deze resultaten worden verderop in dit hoofdstuk besproken in de sectie over toekomstperspectieven.

Contaminatie van kruidenpreparaten

Naast de aanwezigheid van toxische stoffen in kruidenpreparaten die van nature in de gebruikte kruideningrediënten van deze preparaten voorkomen, is contaminatie van kruidenpreparaten eveneens een reden tot zorg. De veiligheid van levensmiddelen met kruideningrediënten kan in gevaar gebracht worden door opzettelijke of toevallige contaminatie met een reeks aan stoffen waaronder bijvoorbeeld polycyclische aromatische koolwaterstoffen (PAK's) en metalen zoals lood. In traditionele geneeswijzen worden met name kruidenpreparaten toegepast, hoewel dierlijke producten en mineralen ook worden gebruikt [14]. In meerdere traditionele geneeswijzen uit Azië zoals Ayurveda, Unani-Tibb, Siddha, TCG en de Traditionele Tibetaanse Geneeswijze (TTG) worden voor therapeutische doeleinden in kruidenpreparaten opzettelijk ingrediënten gebruikt die significante hoeveelheden kwik, arseen of lood bevatten. Hoewel deze ingrediënten algemeen als veilig beschouwd worden in deze traditionele geneeswijzen hebben ze wereldwijd vergiftigingen veroorzaakt. Het doel van het onderzoek dat in Hoofdstuk 5 van dit proefschrift besproken wordt, was daarom het bepalen van de gehalten aan kwik, arseen en lood in Aziatische TKP's op de Nederlandse markt. In totaal werden tussen 2004 en 2007, 292 TKP's bemonsterd die worden toegepast in Ayurveda, TCG and TTG. De monsters waren met name TKP's met meerdere ingrediënten zoals kruiden en mineralen. Uit de etikettering van minder dan 20% van de TKP's kon worden opgemaakt dat kwik, arseen of lood aanwezig zou kunnen zijn. Deze elementen werden aangetoond met een ICP-MS-methode in 186 (64%) van 292 TKP's. Voor elke TKP werd de geschatte wekelijkse innamehoeveelheid aan kwik, arseen en lood berekend op basis van de analytisch bepaalde concentratie en de aanbevolen gebruikshoeveelheid van het product. Gebruik van 59 TKP's (20%) zou waarschijnlijk resulteren in innameniveaus van deze elementen die significant hoger waren dan veiligheidslimieten. Van deze 59 TKP's overschreden innameschattingen voor 50 TKP's op significante wijze veiligheidslimieten (range 1,4-1747 mg/week); innameschattingen voor 26 TKP's overschreden significant de veiligheidslimiet voor arseen (range 0,53-427 mg/week) en innameschattingen voor 8 TKP's waren significant hoger dan de veiligheidslimiet voor lood (range 2,6-192 mg/week). Geconcludeerd kan worden dat de gehalten aan kwik, arseen en lood in TKP's die in Ayurveda, TCG en TTG worden toegepast redenen tot zorg blijven die strikte controle vereisen.

Het onderzoek naar lood, kwik en arseen in Aziatische TKP's gepresenteerd in Hoofdstuk 5 werd in 2008 voortgezet. De resultaten van die studie wezen niet uit dat de veiligheid van deze producten in dat jaar in aanzienlijke mate was toegenomen ten opzichte van de resultaten uit voorgaande jaren. In 2008 werd kwik,

arseen en lood in 60 (77%) van 78 Aziatische TKP's gevonden. Vier van deze preparaten zouden bij gebruik volgens de gebruiksaanwijzing leiden tot kwik of loodinnames boven de veiligheidslimieten die in Hoofdstuk 5 zijn geselecteerd. Op een totaal van 28 TKP's waarin kwik was gevonden, overschreed het kwikgehalte van 27 TKP's de recent voor voedingssupplementen vastgestelde Europese maximumgehalte aan kwik van 0,1 mg/kg. Het loodgehalte van 36 TKP's lag boven het nieuwe Europese maximumgehalte aan lood in voedingssupplementen van 3,0 mg/kg op een totaal van 56 TKP's waarin lood was aangetroffen [15].

Recent heeft EFSA het risico van de blootstelling aan lood en arseen uit de voeding beoordeeld en uit deze risicobeoordelingen bleek dat lood en arseen op de huidige blootstellingsniveaus uit de voeding zorgwekkender zijn dan eerder was vastgesteld. Deze vaststelling heeft ook gevolgen voor de risico's van Aziatische TKP's met lood of arseen zoals deze in Hoofdstuk 5 zijn ingeschat. Het EFSA Panel voor contaminanten in de voedselketen (Panel on Contaminants in the Food Chain - CONTAM) concludeerde in zijn Wetenschappelijke Opinie over arseen in de voeding van oktober 2009 dat de voorlopige grenswaarde voor de toelaatbare wekelijkse inname van arseen van 15 µg per kg lichaamsgewicht (µg/kg lg) vastgesteld door het expertcomité voor levensmiddelenadditieven van de FAO/WHO (Joint FAO/WHO Expert Committee on Food Additives - JECFA) niet langer meer voldoet. Het CONTAM Panel identificeerde een reeks waarden voor de 95% betrouwbaarheidsondergrens van de 'benchmark dose' voor 1% extra risico (BMDL01) voor gebruik bij de karakterisatie van de risico's van anorganisch arseen. De BMDL01-waarden voor de relevante gezondheidseffecten (huidlaesies, huid-, blaas- en longkanker) varieerden van 0,3 tot en met 8 µg/kg lg/dag [16]. In onze studie naar arseen, lood en kwik in Aziatische TKP's die voor 2008 zijn bemonsterd (Hoofdstuk 5) hebben we een TDI van 1,0 µg/kg lg/dag gebruikt die is vastgesteld door het RIVM. Deze TDI is gebaseerd op de JECFA PTWI waarop een veiligheidsfactor van 2 was toegepast om te corrigeren voor observatiefouten in epidemiologische studies [17]. De TDI die door het RIVM voor anorganisch arseen was vastgesteld, bevindt zich tussen de lagere waarden van de reeks van BMDL01-waarden die door het CONTAM panel zijn geïdentificeerd. In onze studie die in Hoofdstuk 5 is besproken, werden in 26 van 292 Aziatische TKP's arseenconcentraties gevonden die bij aanbevolen gebruik zouden leiden tot innamen die significant boven de TDI van het RIVM zouden liggen. Daarnaast werd in 77 andere van de 292 TKP's ook arseen gevonden maar in concentraties die bij het op het etiket aanbevolen gebruik niet zouden leiden tot innamen die significant boven de TDI van 1,0 µg/kg lg/dag zouden liggen. Voor het merendeel van de monsters in deze groep bestaat een veiligheidsmarge (margin of safety) van een orde van grootte van 1 of 2 tussen de geschatte arseeninnameniveaus voor deze monsters en de RIVM TDI. Omdat de TDI in de reeks ligt van BMDL01-waarden die geïdentificeerd zijn door het CONTAM panel kan deze marge eveneens als representatief beschouwd worden voor de reeks van BMDL01-waarden die door het CONTAM Panel zijn geïdentificeerd.

Doordat in de Europese of Nederlandse levensmiddelenwetgeving maximumgehalten aan arseen in voedingssupplementen ontbreken, moet de VWA bewijzen dat een supplement met een relatief hoog arseengehalte schadelijk is voor de gezondheid of ongeschikt voor menselijke consumptie voordat handhavingsmaatregelen kunnen worden genomen. Het in de handel brengen van producten die schadelijk zijn voor de gezondheid of die ongeschikt zijn voor menselijke consumptie is verboden in de Algemene

Levensmiddelenverordening (Europese Verordening (EG) Nr. 178/2002) [18]. De eisen uit deze verordening zijn direct van toepassing in Nederland. Naar aanleiding van de Opinie over arseen in de voeding van het CONTAM Panel moet overwogen worden of de TDI voor anorganisch arseen die door het RIVM is vastgesteld nog gebruikt kan worden om te toetsen of een voedingssupplement met een relatief hoog arseengehalte nog veilig gebruikt kan worden. Om de handhaafbaarheid van de voedselveiligheid van voedingssupplementen te verbeteren moet het vaststellen van maximumgehalten aan arseen in deze producten overwogen worden.

Daarnaast gaf het CONTAM Panel aan dat voedingssupplementen met zeewier behoren tot de groep van levensmiddelen waarin de hoogste totaal-arseengehalten gemeten waren. Dit is met name relevant voor verschillende zeewiersoorten zoals hijiki (*Hizikia fusiforme*) dat hoge totaal-arseengehalten kan bevatten waarvan tot 60% uit anorganisch arseen bestaat naast de eveneens aanwezige organische arseenverbindingen die toxicologisch gezien minder zorgwekkend zijn [16, 19]. In andere zeewiersoorten bestaat maar een gering deel van het totaal-arseengehalte uit anorganisch arseen [19]. Het CONTAM Panel beklemtoonde tevens dat er behoefte is aan gegevens over de speciatie van arseen in verschillende levensmiddelen zodat risicoschattingen daarmee verfijnd kunnen worden [16].

Het CONTAM Panel publiceerde in april 2010 een Opinie over de risicoschatting van lood in voeding. Het Panel concludeerde in zijn Wetenschappelijke Opinie over lood in voeding dat de PTWI van 25 µg/kg lg vastgesteld door JECFA niet meer voldoet. Omdat er geen bewijs was voor het bestaan van een drempelwaarde bij het optreden van een aantal effecten zoals neurotoxiciteit tijdens de ontwikkeling en nefrotoxiciteit bij volwassenen, was het Panel van mening dat het niet juist is om een PTWI vast te stellen. Geconcludeerd werd dat voor lood de huidige blootstellingsniveaus aanleiding tot zorg kunnen geven met betrekking tot effecten op de ontwikkeling van het zenuwstelsel bij zuigelingen, kinderen en zwangere vrouwen. Maatregelen om kinderen en vrouwen in de vruchtbare leeftijd te beschermen tegen een potentieel risico van effecten op de ontwikkeling van het zenuwstelsel zal tevens bescherming bieden tegen alle andere schadelijke effecten van lood in de totale populatie. Het Panel leidde een BMDL01-innameniveau af van 0,50 µg/kg lg/dag voor effecten op de ontwikkeling van het zenuwstelsel [20]. In ons onderzoek naar arseen, lood en kwik in Aziatische TKP's (Hoofdstuk 5) hebben we een TDI van 3,6 µg/kg lg/dag gebruikt die was vastgesteld door het RIVM en die direct gebaseerd was op de JECFA PTWI voor lood [17]. Omdat EFSA niet langer van mening is dat de JECFA PTWI voor lood voldoet, is het raadzaam dat het gebruik van de daaraan gelijkwaardige RIVM TDI door de VWA voor de beoordeling van levensmiddelen waarvoor nog geen wettelijke maximumniveaus bestaan, wordt heroverwogen.

Het kwik dat in Ayurvedische TKP's werd aangetroffen zoals gerapporteerd in Hoofdstuk 5 kan het gevolg zijn van de opzettelijke toevoeging van kwik in Ayurveda aan verschillende TKP's in de vorm van preparaten die bhasma's genoemd worden. Gesteld wordt dat metalen of metalloïden in bhasma's ontgift zijn door intensieve behandelingen. De toxiciteit van kwik hangt af van de chemische vorm waarin het zich bevindt [21, 22]. De giftigheid van elementair kwik, aangeduid als Hg(0), is bij orale blootstelling gering terwijl anorganische en organische kwikverbindingen bij orale blootstelling aanmerkelijk giftiger zijn [22, 23]. Zoals beschreven in Hoofdstuk 5 hebben we 242 Ayurvedische TKP's op kwik onderzocht en het bleek daarbij dat het

gebruik van 19% van deze preparaten volgens het doseringsadvies zou leiden tot blootstellingsniveaus voor kwik boven de toxicologische veiligheidslimiet voor anorganisch kwik. Het doel van het onderzoek dat in Hoofdstuk 6 wordt besproken, is het vaststellen of het kwik in deze Ayurvedische preparaten (gedeeltelijk) aanwezig is als Hg(0), wat in dat geval een argument zou zijn om de risicoschatting waarin limieten voor anorganisch kwik zijn gebruikt te heroverwegen. Voor dit onderzoek hebben we een methode ontwikkeld die de detectie van Hg(0) in deze Ayurvedische TKP's mogelijk maakt. Daarop hebben we onderzoek gedaan naar de aanwezigheid van Hg(0) in 19 Aziatische TKP's waarvan het onderzoek in Hoofdstuk 5 uitwees dat de inname van kwik bij het voorgeschreven gebruik de veiligheidslimiet voor anorganisch kwik zou overschrijden. Uit de resultaten kan worden opgemaakt dat in 11 van de onderzochte 19 TKP's al het aangetroffen kwik afkomstig is van andere vormen dan Hg(0). Extractie van de andere 8 TKP's met 7M HNO_3 liet zien dat het meeste kwik uit deze monsters (69-99%) niet geëxtraheerd kon worden met 7M HNO_3. Omdat was aangetoond dat Hg(0) efficiënt geëxtraheerd kan worden met 7M HNO_3 wijst dit resultaat er op dat kwik in andere vormen dan Hg(0) aanwezig is. Geconcludeerd werd dat in alle TKP's het merendeel van het kwik niet aanwezig is in de elementaire vorm waarvan de giftigheid relatief laag is. Tevens werd geconcludeerd dat het kwik in Ayurvedische TKP's waarschijnlijk aanwezig is in anorganische vorm en dat daarom het schatten van de gerelateerde risico's op basis van veiligheidslimieten voor anorganische vorm gerechtvaardigd is. Tenslotte werden geen additionele risicobeoordelingen van anorganisch kwik door nationale of internationale expert comités gevonden.

De metalen en metalloïden die in Aziatische TKP's werden aangetroffen, zijn hoogstwaarschijnlijk het gevolg van de opzettelijke toevoeging en dit opzicht verschillen deze stoffen hier van contaminanten zoals polycyclische aromatische koolwaterstoffen (PAK's) die onbedoeld voedsel kunnen vervuilen als gevolg van verschillende processen. PAK's kunnen tevens voorkomen in voedingssupplementen en de Nederlandse Warenwet bevat sinds januari 2006 een maximumniveau van 10 µg/kg benzo[a]pyreen voor kruidenpreparaten en een maximumniveau van 2 µg/kg voor supplementen zonder kruideningrediënten [24].

EFSA heeft 16 prioriteits-PAK's geïdentificeerd die genotoxisch en carcinogeen zijn waarvan 8 prioriteits-PAK's (PAK8) of 4 van deze (PAK4) goede indicators zijn voor de toxiciteit en het voorkomen van PAK's in de voeding. Het onderzoek dat in Hoofdstuk 7 is beschreven, heeft het bepalen van benzo[a]pyreen en andere EFSA prioriteits-PAK's in verschillende categorieën van voedingssupplementen met kruidenpreparaten en andere ingrediënten ten doel. In 2003 tot en met 2007 overschreed benzo[a]pyreen de bepaalbaarheidsgrens in 553 (44%) van 1258 supplementen met een 'lower bound' gemiddelde van 3,37 µg/kg. In 2008 en 2009 werden benzo[a]pyreen en 12 andere EFSA prioriteits-PAK's bepaald in 333 voedingssupplementen. Het benzo[a]pyreengehalte overschreed de bepaalbaarheidsgrens in 210 (63%) van de voedingssupplementen met een lower bound gemiddelde van 5,26 µg/kg. Lower bound gemiddelden van PAK4 en PAK8 (-indeno[1,2,3-cd]pyreen) waren 33,5 µg/kg en 40,5 µg/kg, respectievelijk. Supplementen met resveratrol, *Ginkgo biloba*, sint-janskruid en propolis vertoonden relatief hoge PAK's-niveaus in 2008 en 2009. Vóór 2008 bevatten supplementen met deze ingrediënten en ook die met dong quai, groene thee of valeriaan eveneens relatief hoge gehalten aan benzo[a]pyreen. Gemiddeld genomen zal de inname van PAK's door het gebruik van voedingssupplementen zich aan de onderkant bevinden van een reeks van bijdragen van hoofdcategorieën van

levensmiddelen aan de blootstelling aan PAK's, hoewel de bijdrage van individuele voedingssupplementen aan de blootstelling aan PAK's significant kan zijn. Regelmatige controle van de gehalten aan PAK's in voedingssupplementen kan een geschikt instrument zijn om de inname van PAK's uit voeding verder te verlagen. Het was interessant dat in totaal bij 192 (58%) van de supplementen uit het onderzoek in 2008 en 2009 (n=333) de PAK4 gehalten lager lagen dan 10 µg/kg en dat in 99 (30% van het totaal) van deze 192 supplementen de PAK4 gehalten onder de bepalingsgrens lagen. Daarnaast werden in elke subcategorie van voedingssupplementen uit het onderzoek supplementen gevonden zonder PAK's of met relatief lage gehalten aan PAK's.

Alles samengenomen kan gesteld worden op basis van de onderzoeken naar contaminatie van kruidenpreparaten op de Nederlandse markt met aristolochiazuren, lood, kwik, arseen en PAK's waarvan de analytische data in dit proefschrift zijn gepresenteerd dat contaminatie van kruidenpreparaten met PAK's het meest voorkomt, gevolgd door contaminatie met kwik, arseen of lood, en met aristolochiazuren op de laatste plaats. De toxiciteit van deze verschillende substanties kan niet makkelijk met elkaar vergeleken worden. Wanneer echter voor elk van deze contaminanten de effecten van blootstelling aan de bovenkant van de in dit proefschrift geschatte blootstellingsniveaus gedurende een bepaalde tijdsduur vergeleken zouden worden, dan zouden aristolochiazuren waarschijnlijk het meest schadelijk zijn voor de gezondheid, gevolgd door de toxische effecten van lood, arseen, kwik en PAK's, in die volgorde. Echter, het feit dat aristolochiazuren alleen werden aangetroffen in een klein segment van het aanbod van TCG-winkels en dat aanzienlijk meer Aziatische TKP's op de markt gevonden werden die lood, arseen of kwik bevatten, doet vermoeden dat in Nederland op dit moment de kans op schadelijke effecten groter is bij Aziatische TKP's met relatief hoge gehalten aan lood, arseen of kwik. Doordat de in Hoofdstuk 7 geschatte innameniveaus van PAK's uit voedingssupplementen in dezelfde orde van grootte liggen als de blootstellingniveaus aan PAK's uit levensmiddelen die voor de Europese Unie zijn vastgesteld, zijn de geassocieerde effecten op de gezondheid van een lagere orde dan de effecten van aristolochiazuren, lood, kwik en arseen. Evenwel, omdat verschillende PAK's genotoxisch en carcinogeen zijn en blootstelling aan PAK's uit de voeding voor consumenten met hoge blootstellingsniveaus een mogelijke reden tot zorg is, zouden inspanningen verricht moeten worden om de blootstelling aan PAK's uit voedingssupplementen te verlagen en zo een verdere daling van de blootstelling uit de voeding te kunnen bewerkstelligen.

Toekomstperspectieven

De Europese Commissie concludeerde in 2008 dat het vaststellen van specifieke Europese regels voor andere stoffen dan vitaminen en mineralen voor gebruik in voedingssupplementen niet gerechtvaardigd is [25]. Dit betekent dat het niet waarschijnlijk is dat er in de nabije toekomst een Europese pendant van het Nederlandse Warenwetbesluit Kruidenpreparaten zal komen. Verordening (EG) Nr. 1925/2006 stelt in artikel 8 een procedure vast die gebruikt dient te worden voor gevallen waarin een andere stof dan vitaminen of mineralen (bijvoorbeeld een kruidenpreparaat) een potentieel risico voor de consument zou kunnen vormen. De Commissie kan op basis van een risicobeoordeling van EFSA of informatie van een Lidstaat het besluit nemen om het gebruik van de stof

in levensmiddelen te beperken of te verbieden. De veiligheid van kruideningrediënten kan beoordeeld worden met de methode die EFSA ontwikkeld heeft voor de risicobeoordeling van kruidensubstanties en kruidenpreparaten in combinatie met het EFSA Compendium van kruidensubstanties waarvan gerapporteerd is dat ze toxische, verslavende of psychotrope stoffen bevatten of andere stoffen die aanleiding voor bezorgdheid geven. Er zijn nog geen besluiten genomen op basis van deze procedure. Het is nog niet duidelijk in welke mate de Commissie gebruik zal maken van de procedure in artikel 8 van Verordening (EG) Nr. 1925/2006 om het gebruik van bepaalde kruidenpreparaten in levensmiddelen te beperken of te verbieden.

EFSA wees er reeds in 2004 op dat het gebruik van kruidensubstanties en kruidenpreparaten in levensmiddelen aan het toenemen was [2]. De potentiële risico's van kruidenpreparaten zullen daarom ook in betekenis toenemen. Omdat het niet waarschijnlijk is dat binnen afzienbare tijd nieuwe Europese regelgeving voor het gebruik van kruidensubstanties en kruidenpreparaten in voedingssupplementen wordt vastgesteld, is het verstandig om regelgeving zoals het Warenwetbesluit Kruidenpreparaten bij te blijven werken om Nederlandse consumenten te beschermen tegen ernstige risico's van het gebruik van kruidenpreparaten. Wanneer een aanpassing van het Kruidenbesluit in gang wordt gezet is het aanbevelenswaardig dat hierbij risicobeoordelingen en aanbevelingen over kruidenpreparaten door nationale en Europese risicobeoordelaars zoals het RIVM, EMA en EFSA in aanmerking worden genomen.

Verschillende risicobeoordelingsorganen hebben voor bepaalde kruidenpreparaten aanbevelingen gedaan om waarschuwingsteksten op de etikettering te plaatsen. EMA heeft bijvoorbeeld waarschuwingsteksten vastgesteld voor interacties tussen kruidengeneesmiddelen met sint-janskruid en andere geneesmiddelen [26]. Omdat sint-janskruid ook in voedingssupplementen toegepast wordt, kunnen deze waarschuwingsteksten van belang zijn voor alle sint-janskruidproducten voor oraal gebruik die op de markt worden aangeboden. Daarnaast heeft een aantal risicobeoordelaars aanbevolen om op preparaten met zilverkaars (*Actaea racemosa* of *Cimicifuga racemosa*) een waarschuwingstekst te plaatsen over het vermogen van het kruid om leverschade te veroorzaken [27]. Overwogen moet worden om in het Warenwetbesluit Kruidenpreparaten een verplichting op te nemen om waarschuwingsteksten te plaatsen op de etikettering van bepaalde kruidenpreparaten die aanleiding tot zorg geven maar die veilig gebruikt zouden kunnen worden als de consument geïnformeerd wordt over hoe specifieke gezondheidseffecten vermeden kunnen worden. Het zou echter raadzaam zijn om vast te stellen voor welke gezondheidseffecten deze aanpak geschikt is. Hoewel het ongebruikelijk is in Nederland dat op de etikettering van levensmiddelen waarschuwingen staan, zijn er verplichte waarschuwingsteksten vastgesteld voor levensmiddelen met bepaalde gehalten aan glycyrrhizinezuur. Op dit moment geldt deze verplichting niet voor kruidenpreparaten zoals voedingssupplementen met kruideningrediënten. Evenwel zou overwogen moeten worden om deze verplichting ook te laten gelden voor kruidenpreparaten met *Glycyrrhiza*-soorten die glycyrrhizinezuur bevatten.

Daarnaast is het belangrijk om op te merken dat het plaatsen van een waarschuwingstekst op de etikettering van een product dat in verband is gebracht met bepaalde schadelijke gezondheidseffecten geen garantie is voor het uitblijven van verdere gevallen van gezondheidsschade. Dit wordt geïllustreerd door de verschillen in resultaten van diverse methoden waarmee wereldwijd getracht wordt om consumenten te

beschermen tegen schadelijke effecten van kava kava. Nadat het gebruik van dit kruid in verband werd gebracht met leverschade werden kruidenpreparaten met kava kava in 2003 verboden in Nederland. In de Verenigde Staten werd kava kava niet van de markt gehaald. De fabrikanten van kruidenpreparaten in de Verenigde Staten plaatsten daarentegen waarschuwingsteksten over mogelijke leverschade op de etikettering van deze preparaten en de Food and Drug Administration (FDA) publiceerde tevens waarschuwingen over dit kruid. In weerwil van deze maatregelen bleef de FDA rapportages van levertoxiciteit ontvangen. Australië voerde eveneens waarschuwingsteksten in die wezen op het verband tussen kava kava en het in zeldzame gevallen optreden van leverschade, maar ook hier bleven de rapportages van levertoxiciteit aanhouden [28].

EFSA concludeerde in 2004 dat zowel de groei in de verkoopvolumes in de Europese Unie van producten uit Azië of andere gebieden als de toenemende verspreiding van verkooplocaties van producten die gebruikt worden in traditionele geneeswijzen vragen om een meer formele beoordeling van die producten voordat ze op de markt gebracht worden. Daarnaast vragen deze ontwikkelingen volgens EFSA ook om strengere controles dan de sporadische keuringen en analyses van producten die al op de markt zijn en die meestal uitgevoerd worden door individuele nationale en lokale autoriteiten [2]. In de huidige Nederlandse en Europese wet- en regelgeving voor levensmiddelen met kruideningrediënten zoals besproken in Hoofdstuk 2 zijn geen bepalingen opgenomen die het mogelijk maken om alle producten die op de markt gebracht worden, voorafgaande aan de marktintroductie aan een strenge beoordeling te onderwerpen. Het is echter wel mogelijk om de frequentie te verhogen van het onderzoek naar natuurlijke gifstoffen uit kruiden en contaminanten in voedingssupplementen die al op de markt zijn. Deze benadering kan leiden tot een verbetering van de veiligheid van deze producten, hoewel dit een langdurig proces kan zijn. Er zijn bijvoorbeeld aanwijzingen dat de handhavingsacties van de VWA hebben geleid tot een afname in het aantal TKP's met aristolochiazuren die in het winkelkanaal worden aangeboden. In 2008 bemonsterde de VWA 77 Chinese TKP's voor onderzoek op aristolochiazuren en in één TKP werden sporen van aristolochiazuren gevonden [15]. Daarentegen werden in 25 van 190 Chinese TKP's die vanaf 2002 tot en met 2006 waren bemonsterd aristolochiazuren gevonden (Hoofdstuk 4 van dit proefschrift). Conclusies over een mogelijke verlaging van de kans op contaminatie met aristolochiazuren van kruidenpreparaten die in Nederland in het winkelkanaal worden aangeboden kunnen echter alleen getrokken worden op basis van de resultaten van meerdere jaren van aanvullend marktonderzoek. Op basis van de toekomstige resultaten kan dan beoordeeld worden of een verbod op het gebruik in kruidenpreparaten van *Asarum*-soorten en de planten die mogelijk verwisseld kunnen worden met *Aristolochia*-soorten noodzakelijk is. Een andere reden om de veiligheid van het gebruik van *Asarum*-soorten in kruidenpreparaten op de markt te beoordelen, is de mogelijke aanwezigheid van alfa-asaron, een stof die bij zoogdieren kankerverwekkend is [29]. Tevens moet in aanmerking genomen worden dat consumenten in aanraking kunnen komen met producten met aristolochiazuren via kanalen zoals het internet wat het toezicht door de overheid kan bemoeilijken. Alles samengenomen is het essentieel dat een exploitant van een levensmiddelenbedrijf dat TKP's op de markt brengt die in theorie aristolochiazuren zouden kunnen bevatten door het opzettelijk toepassen van planten uit de familie van de *Aristolochiaceae* of door verwisseling van de

bedoelde kruideningrediënten met *Aristolochia* spp., beheersmaatregelen opneemt in zijn kwaliteitssysteem (Hazard Analysis Critical Control Points - HACCP) om dit gevaar te borgen.

De Opinies van het CONTAM Panel over arseen en lood [16, 20] zijn van belang voor de handhaving van de veiligheid van kruidenpreparaten en andere voedingssupplementen. Vastgesteld moet worden of de TDI van het RIVM voor anorganisch arseen en die voor lood nog geschikt zijn voor de beoordeling of een voedingssupplement met een relatief hoog gehalte aan arseen of lood veilig gebruikt kan worden. Omdat maximumgehalten aan arseen in voedingsupplementen zouden kunnen helpen bij de handhaving van de veiligheid van deze producten, zou het vaststellen van dergelijke maximumgehalten overwogen moeten worden. Tevens is data over de speciatie van arseen en kwik in verschillende levensmiddelen nodig om risicobeoordelingen verder te kunnen verfijnen [16].

Enkele resultaten van ons onderzoek naar PAK's in voedingssupplementen (Hoofdstuk 7 van dit proefschrift) kunnen van betekenis zijn voor de geplande vaststelling van maximumgehalten aan PAK's in voedingssupplementen in de Europese voedselveiligheidswetgeving. Zo waren de PAK4-waarden in bijna 60% van de supplementen uit het onderzoek in 2008 en 2009 lager dan 10 µg/kg. Daarnaast werden in alle subcategorieën van voedingssupplementen in het onderzoek supplementen gevonden met gehalten aan PAK's die niet bepaalbaar of relatief laag waren. Dit doet vermoeden dat contaminatie van voedingssupplementen met PAK's vermijdbaar is.

Conclusie

Op basis van het onderzoek dat in dit proefschrift is beschreven, kan geconcludeerd worden dat bij kruidenpreparaten niet geldt dat 'natuurlijk' hetzelfde is als 'veilig'. Met name de onderzoeksresultaten die in Hoofdstukken 4 en 5 zijn besproken, tonen aan dat slechts het feit dat een kruidenpreparaat op de Nederlandse markt verkrijgbaar is, niet garandeert dat het product veilig is en dat het geen schadelijke effecten op de gezondheid zal hebben. Rapportages van nationale en Europese risicobeoordelingsorganen zoals het RIVM, EMA en EFSA en andere bronnen uit de literatuur die in Hoofdstukken 2 en 3 zijn besproken, tonen aan dat andere kruidenpreparaten dan de preparaten waarvoor op dit moment specifieke bepalingen zijn opgenomen in de Nederlandse voedselveiligheidswetgeving eveneens op toxicologische gronden reden tot zorg vormen. Het is van belang dat consumenten zich realiseren dat het risico van bijwerkingen toeneemt wanneer kruidenpreparaten buiten de reguliere winkelkanalen om worden aangeschaft, zoals het geval is wanneer preparaten gekocht worden via het internet of bij winkels met producten uit traditionele geneeswijzen, smartshops of bij vergelijkbare winkeltypen. Hoewel levensmiddeleninspectiediensten relatief veel capaciteit inzetten op de handhaving van de voedselveiligheid in de voedingssupplementenbranche, ligt de primaire verantwoordelijkheid voor de veiligheid van voedingssupplementen bij de exploitanten van bedrijven die deze levensmiddelen op de markt brengen. Het is van essentieel belang dat ondernemers die kruidenpreparaten op de markt hebben gedetailleerde kennis hebben van de gevaren die deze producten met zich meebrengen. Tevens moeten ondernemers in kwaliteitssystemen (HACCP) beheersmaatregelen opnemen waarmee alle relevante gevaren voorkomen,

geëlimineerd, dan wel tot een aanvaardbaar niveau gereduceerd kunnen worden. Het feit dat een levensmiddeleninspectiedienst nog geen producten van een exploitant van een levensmiddelenbedrijf heeft onderzocht op de aanwezigheid van bepaalde stoffen die schadelijk kunnen zijn, of heeft beoordeeld of de ondernemer maatregelen heeft genomen om deze gevaren te voorkomen, elimineren, dan wel tot een aanvaardbaar niveau te reduceren, betekent niet dat deze gevaren geen maatregelen vereisen. Tenslotte moet in aanmerking worden genomen dat de Europese Commissie geconcludeerd heeft dat nieuwe Europese wetgeving voor kruidensubstanties en kruidenpreparaten die in voedingsupplementen gebruikt worden niet nodig is en dat de huidige wetgeving volstaat om het gebruik in levensmiddelen van specifieke toxische substanties zoals bepaalde kruidenpreparaten te beperken of te verbieden. Omdat het nog niet duidelijk is in welke mate de Europese Commissie gebruik zal maken van deze mogelijkheden is het verstandig om het Nederlandse Warenwetbesluit Kruidenpreparaten en andere nationale wetgeving bij te blijven werken om Nederlandse consumenten te beschermen tegen ernstige risico's van het gebruik van kruiden in levensmiddelen zoals kruidenpreparaten.

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CURRICULUM VITAE

Martijn Johan Martena was born on 13th of April 1975 in Nieuwleusen, The Netherlands. In 1999, he obtained his MSc degree in Human Nutrition at Wageningen Agricultural University, specializing in nutrition and health. As part of this study he conducted research projects on the uptake of beta-carotene in mixed lipid-bile salt micelles and the determination of selected phytochemicals. The latter was conducted at the Institute for Biological Chemistry and Nutrition of the University of Hohenheim in Stuttgart. Owing to these research projects he developed a lasting interest in the effects of active substances in plants on health. After obtaining his degree, he worked in 2000 at two Internet startup companies selling food supplements, where he was responsible for the scientific content of the companies' websites. Early 2001, he left e-commerce to work for his current employer, the Dutch Food and Consumer Product Safety Authority (VWA). His activities at the VWA are aimed at the safety of food supplements, herbal preparations and other health foods, food safety law, the health claims legislation and enforcement. In this capacity he started surveys into the presence of naturally occurring toxins and contaminants in herbal preparations. Using the results of these investigations, he began working in 2005 on a PhD project under supervision of prof. Dr. ir. I.M.C.M. Rietjens of the Department of Toxicology of Wageningen University while continuing his work at the VWA. In addition, he finished the Postdoctoral Education in Toxicology in 2007. In that same year he was appointed member of the Committee for Risk assessment of novel drugs by the Minister of Health. In 2008, he participated in the EFSA Scientific Cooperation working group on Botanicals and botanical preparations. Additionally, he takes part in the National Network Drugs Expertise and other governmental working groups.

LIST OF PUBLICATIONS

Published manuscripts

- Martena MJ, van der Wielen JCA, Klerx WNM, de Groot HN, Rietjens IMCM, Konings EJM. Monitoring of mercury, arsenic, and lead in traditional Asian herbal preparations on the Dutch market and estimation of associated risks. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2010, 27, 190-205.
- Martena MJ, van der Wielen JCA, van de Laak LFJ, Konings EJM, Groot HN, Rietjens IMCM. Enforcement of the ban on Aristolochic acids in Chinese traditional herbal preparations on the Dutch market. Anal Bioanal Chem. 2007, 389, 263-275.
- van der Wielen JC, Jansen JT, Martena MJ, De Groot HN, In 't Veld PH. Determination of the level of benzo[a]pyrene in fatty foods and food supplements. Food Addit Contam. 2006, 23, 709-714.
- Rietjens IMCM, Martena MJ, Boersma MG, Spiegelenberg W, Alink GM. Molecular mechanisms of toxicity of important food-borne phytotoxins. Mol Nutr Food Res. 2005, 49, 131-158.
- Andlauer W, Martena MJ, Fürst P. Determination of selected phytochemicals by reversed-phase high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. J Chromatogr A.1999, 849, 341-348.

Manuscripts submitted or in preparation

- Martena MJ, Grutters MMP, Konings EJM, de Groot HN, Rietjens IMCM. Monitoring of Polycyclic Aromatic Hydrocarbons (PAH) in food supplements with botanicals and other ingredients on the Dutch market [submitted for publication].
- Martena MJ, de Haan E, Klerx WNM, Konings EJM, Rietjens IMCM. Detection of elemental mercury in selected traditional Ayurvedic herbal preparations [in preparation].

Abstracts

- Martena MJ, van der Wielen JCA, Klerx WNM, de Groot HN, Rietjens IMCM. Heavy metal ingredients of traditional Asian herbal preparations. Abstracts of the Dutch toxicology days, 13th-14th June 2006. Chem.- Biol Interact. 2006, 161, 165-175.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific training activities

Courses

PET: risk assessment	2005
PET: organ toxicology	2005
PET: food toxicology	2005
PET: medical/ forensic toxicology	2005
PET: laboratory animal science	2006
PET: cell toxicology	2006
PET: molecular toxicology	2006
PET: mutagenesis & carcinogenesis	2007
PET: ecotoxicology	2007

Meetings

Netherlands Society of Toxicology: PhD toxicology days, Wageningen (poster presentation)	2006
EU-TAIEX: workshop on Food Supplements, Warsaw, Poland (oral presentation)	2006
EU-TAIEX: workshop on market control of food supplements and foodstuffs for particular nutritional uses, Vilnius, Lithuania (oral presentation)	2007
Botanical Forum: workshop on botanical food supplements	2007
EU-TAIEX: workshop on foodstuffs for particular nutritional uses, Zagreb, Croatia (oral presentation)	2008
SANA: workshop on botanicals, Bologna, Italia (oral presentation)	2009
Netherlands Society of Toxicology: workshop “Innovative Toxicity Testing”, Veldhoven	2009

General courses

WBS: scientific writing	2003
EU-DG SANCO: HACCP course	2009
Boertien Groep: time management	2007, 2008
Kessels & Smit: personal effectiveness and knowledge management	2009

Optionals

Preparation of research proposal	2005
VWS: Committee for risk assessment of novel drugs (member)	2007, 2008 & 2009
EFSA: Scientific Cooperation working group on botanicals and botanical preparations (member)	2009

Abbreviations

EFSA	European Food Safety Authority
EU-DG SANCO	European Commission, Directorate-General for Health and Consumers
EU-TAIEX	European Commission, Directorate-General Enlargement,Technical Assistance and Information Exchange programme
HACCP	Hazard Analysis Critical Control Points
PET	Postgraduate Education in Toxicology
SANA	International Exhibition of Natural Products
VWS	Dutch Ministry of Health
WBS	Wageningen Bussiness School

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Image on cover: root cross section of a Clematis (Shutterstock)
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