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SCIENCE FOR A SUSTAINABLE DEVELOPMENT

FOOD INTERACTIONS : EFFECTS ON HEALTH, CONSUMER PERCEPTION AND IMPACT ON AGRO-FOOD INDUSTRIES

"FOODINTER"

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FINAL REPORT PHASE 1

FOOD INTERACTIONS : EFFECTS ON HEALTH, CONSUMER PERCEPTION AND IMPACT ON AGRO-FOOD INDUSTRIES

"FOODINTER"

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Table of content

ACRONYMS, ABBREVIATIONS AND UNITS

3-ADON	3-acetyldeoxynivalenol
5-MC	5-Methylchrysene
15-ADON	15-acetyldeoxynivalenol
ADI	Acceptable daily intake
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFSSA	Agence française de sécurité sanitaire des aliments
AhR	Aryl hydrocarbon receptor
ALT	Altenuen
AOH	Alternariol
AME	Alternariol methylether
AMA	Advanced Mercury Analyzer
As	Arsenic
Ba	Baryum
BaA	Benz[a]anthracene
BaP	Benzo[a]pyrene
BbF	Benzo[b]fluoranthène
BcL	Benzo[c]fluorène
Bi	Bismuth
BEAU	Beauvericin
BgP	Benzo[ghi]pérylène
BjF	Benzo[j]fluoranthène
BkF	Benzo[k]fluoranthène
BMDL	Benchmark dose lower confidence limit
Cd	Cadmium
CHR	Chrysene
CPP	Cyclopenta[cd]pyrene
Co	Cobalt
Cr	Chromium
Cu	Copper
DAD	Diode array detection
DAS	Diacetoxyscirpenol
DeP	Dibenzo[ae]pyrène
DhA	Dibenz[ah]anthracène
DhP	Dibenzo[ah]pyrène
DiP	Dibenzo[ai]pyrène
DlP	Dibenzo[al]pyrène
DON	Deoxynivalenol
DRI	Dietary reference intake
EDI	Estimated daily intake
EMEA	European medicines agency
EFSA	European food safety authority
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
FAO	Food and Agriculture organization
FASFC	Federal agency for the safety of the food chain
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
FF	Functional food
FS	Food supplement

FSA	Food Safety Agency
F-X	Fusarenon-X
GL	Glucosinolates
GRH	Glucoraphasatin
GTL	Glucotropaeolin
Hg	Mercury
HPLC/UV-FLD	High performance liquid chromatography/ultraviolet/ fluorescence detection
HT-2	HT-2 toxin
IAEA	Atomic Energy Agency
IC50	Concentration at which 50% of the growth was inhibited
IcP	Indeno[1,2,3-cd]pyrène
ICP-MS	Inductively coupled plasma with mass spectrometer
JECFA	Joint FAO/WHO Experts Committee on Food Additives
LC50	Concentration at which 50 % of the cells are dead
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOQ	Limit of quantification
Mn	Manganese
Mo	Molybdenum
MOA	Mode of action
MOE	Margin of exposure
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MT	Metallothionein
NC	Non compliant
NEO	Neosolaniol
Ni	Nickel
NIST	National institue of standards and technology
NIV	Nivalenol
OTA	Ochratoxin A
PAH	Polycyclic aromatic hydrocarbon
Pb	Lead
PCB	Polychlorinated Biphenyls
RIVM	National institute for public health and the environment (The Netherlands)
RP-LC	reversed-phase liquid chromatography
RRF	relative response factor
Sb	Antimoine
SCF	Scientific Committee on Food
Se	Selenium
SPE	solid phase extraction
STERIG	sterigmatocystin
Sr	Strontium
T-2	T-2 toxin
TCDD	Tetrachlorodibenzodioxin
Ti	Titanium
Tl	Thallium
TMDI	Tolerable maximal daily intake
VDS	Virtual dose sure
WHO	World Health Organization
ZEA	zearalenon
Zn	Zinc

SUMMARY

Food supplements and functional food become of increasingly great interest, as they are now consumed by more and more people These food supplements (e.g. nutrients, vitamins, hormones, amino acids, anti-oxidants,), as well as functional food (e.g. phytosterols or omega-3 fatty acids enriched food) occupy a position between food and drugs. Botanical materials represent a large segment of this class of products (e.g. soy isoflavones, yam or hop extracts).

In many cases, there are still a number of unknowns such as the identification of specific active components and impurities, the effects of processing, the presence of toxic compounds, as well as their absorption and metabolism in the human body.

In the past, the attention of food toxicologists has been focused on the toxicity of single contaminating substances. The interactions between active and potentially toxic substances are poorly documented. Interactions can lead to additive or subtractive or even synergistic effects, which are being studied in FOODINTER.

Furthermore, this project aims to promote the communication between scientists and stakeholders (authorities, producers and consumers). In the field of food consumption, this objective is important because food safety depends not only on production and control, but also on consumption practices and good information must therefore be promoted. It is not only an education plan and the objective is also to promote a dialog between science and society in order to better identify the social preoccupations and need that research has to satisfy.

The objective of this project is thus to contribute to the risk assessment of chemicals, natural compounds and environmental contaminants, present in food supplements which could interact between them or with micro or macronutrients of normal human diet.

Interactions studies have been performed using existing *in vitro* models (based on culture of various cell types, prokaryotes and eukaryotes) with mixture of active substances at concentrations not yet studied until now and very close to the real situation in human nutrition. Extrapolation from the *in vitro* observations to the real risks for human will be attempted.

In phase I of the FOODINTER project, 3 steps were performed:

1. Preliminary information collection
2. Collection of samples, analysis of chemical contaminants and active ingredients, and construction of a data base
3. Biological *in vitro* assays

1. Preliminary information collection

Within this task, several sources of information were consulted in order to gather basic information on food supplements but also in order to prepare the collection of samples on which the experimental studies will be performed. Different approaches were followed such as overview of the Belgian and European legislations, list of products notified in Belgium, overview on the information and choice of products available on the internet and in commercials, overview of the scientific literature dealing with health effects and interactions, contacts with producers and with consumers organized by means of surveys and focus group meetings, etc.

The European Directive 2002/46/EC stated food supplements (FS) as "*foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities*". Examples of FS are ampoules of omega-3, tablets of vitamin A, tablets of multi-vitamin and multi-mineral capsules or capsules of plant extracts such as valerian, garlic,…

The present project will focus more precisely on the botanical preparations marketed as FS.

In Belgium, regulation of FS is grounded on two main Royal Decrees. The first one tackles the issue of Nutrients and their use into food supplements (AR 3/03/92). Among definitions stated in the documents, FS are defined as "*pre-dosed foodstuffs containing one or several nutrients, plants or plant preparations, or any other substance having a physiological or nutritive effect and which goal is to supplement normal diet.*" whereas nutrients are "*nutritive substances needed by the human organism*".

This Royal Decree mentions the notifying process through which a FS has to go in order to be marketed in Belgium. Maximal and minimal limits in terms of % of the Dietary Reference Intake (DRI) are fixed for different nutrients used in FS. Guidelines are detailing the labelling and the advertising of these FS.

The second Royal Decree concerns plants and plant preparations (AR 29/08/1997). It contains a list of dangerous plants whose use for direct consumption or as ingredient of preparation is strictly prohibited. Besides, another list gathers all plants allowed for direct consumption or as ingredient of preparation as long as a notification file has been accepted by federal authorities.

The Food Consumption Study performed by the Scientific Institute of Public Health in 2004 provides us data on FS consumption within the Belgian population (De Vriese et al., 2006). The study showed that 12 % of the population has included FS in their diet.

Consumer opinion was collected via surveys and focus group. From a first analysis of the results, it appeared that : 1) people do not exactly know what kind of preparations can be categorized as food supplements (a lot of hesitation for vitamins and plant extracts), 2) women seem to consume food supplements more often than men, 3) 37% of questioned people do consume food supplements from their own initiative (without medical advice) , 4) the main purpose of consuming food supplements is, according to consumers, to reinforce the immune system of the organism and to fight against tiredness (obviously for vitamins and mineral), 5) a lot of consumers are regular customers but mostly the money spend for buying food supplements is less than 50 Euros, 6) most of the consumers do read the label and are convinced of the beneficial effects as they are described in the label, 7) the majority of the questioned people do believe that food supplements are "natural" but seem to be aware that simultaneous intake of drugs can pose a health risk.

2. Collection of samples, analysis of chemical contaminants and active ingredients and construction of a database

The final selection was made of six different FS all made from one specific plant material:

- **Garlic (G):** Decreases arterial tension; very common botanical product, interactions with drugs
- **Ginkgo biloba (B)**: Improves blood circulation and cerebral oxygenation; very common botanical product, interactions with drugs
- **Sint-John's Wort (W):** Against mild depressions; very common botanical product, interactions with drugs
- **Soy isoflavones (I):** Reduces menopause effects; frequently used; hormonal activity
- **Maca (M):** Increases libido and limit sexual disorders; plant toxins (alkaloids); less studied
- **Black radish (R):** Stimulation of bile secretion and of intestine activity; plant toxins (glucosinolates); less studied

In total, 61 samples were thus collected. They were purchased from 37 companies via internet (36 samples), drugstores (18 samples) and specialized shops (7 samples). 25 are notified in Belgium whilst 36 are not notified (and generally available via the internet). This material was used to perform the analyses of chemical contaminants, of active ingredients as well as for the *in vitro* studies.

A data base has been compiled using all the relevant information on the uses of FS, the nature of their active ingredients, the **contents of active ingredient** s in the FS, the intake of active ingredient according to the recommended doses, the methods of analysis, the **biological studies performed on the active ingredients**, the **chemical contamination** of FS, etc

Analysis of chemical contaminants

Analysis of mineral elements

Seventeen trace elements (As, Ba, Bi, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Se, Sr, Ti, Tl, Zn) were quantified by inductively coupled plasma with mass spectrometer (ICP-MS). Mercury (Hg) was quantified by Advanced Mercury Analyzer (AMA).

There were 10 non compliant (NC) samples with respect to the Belgian legislation for toxic element in FS (7 NC for Pb and 4 NC for Cd; one sample exceeded the norm for both elements).

Analysis of mycotoxins

The target mycotoxins included nivalenol (NIV), deoxynivalenol (DON), neosolaniol (NEO), fusarenon-X (F-X), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), T-2 toxin (T-2), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA), altenuen (ALT), alternariol (AOH), alternariol methylether (AME), fumonisin B1 (FB1), fumonisin B2 (FB2), fumonisin B3 (FB3), zearalenon (ZEA), beauvericin (BEAU), sterigmatocystin (STERIG). They were analyzed using gradient reversed-phase liquid chromatography (RP-LC) with electrospray ionization tandem mass spectrometry (ESI-MS/MS).

The toxins FB1, FB2, FB3 and OTA were detected in some samples. In 2 samples (one of Gingko Biloba and one of Maca), OTA was found at a level above 2 µg/kg (EC norm for wine and grape juice, Regulation 1881/2006/EC). The levels of FB1, FB2 and FB3 were largely below 800 µg/kg (EC norm for the sum of FB1 and FB2 in breakfast cereals, Regulation 1881/2006/EC) in all samples.

Analysis of PAHs

High performance liquid chromatography coupled to an ultraviolet, diode array or fluorescence detector (HPLC/UV-FLD) has been used to detect the 15(+1) EU priority PAHs in the sixty food supplements selected in this project.

The results have shown that S^{t}-John's wort and ginkgo biloba extracts presented the most frequent contaminations and the highest average values for PAHs concentrations.. The most contaminated samples with the sum of the 16 PAHs were generally detected in St-John's wort and ginkgo products, except one sample of soy isoflavones .

From a preliminary risk assessment, we found five that food supplements were health concern (the daily intake from FS could be higher that the tolerable daily intake), one Black radish FS, one Ginkgo biloba FS, two St John's Wort FS and one Gralic FS. All are not notified.

Analysis of organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs) and of polybrominated diphenyl ethers (PBDEs), and dioxins in oily FS

Low amounts of p,p'-DDE or p,p'-DDD (< 10 µg/Kg) were sdetected in 3 garlic samples, but far below the legal limit of 50 µg/Kg for the sum of DDT. Dioxins, PCBs and PBDEs were below the limit of quantification of the assay.

Identification and analysis of active ingredients

Relevant active ingredients were identified from the literature in the 6 categories of food supplements.

These active ingredients are listed below.

St John's wort	Ginkgo biloba	Soy isoflavones	Black Radish	Garlic	Maca
Hypericin	Ginkgolide A	Genistein	L-sulforaphane	Garlic oil	lepidilin A
Hyperforin	Ginkgolide B	Daidzein	DL-sulforaphane	S-allyl cysteine	Lepidlin B
	Ginkgolide C	Glycetein	Glucoraphanin	Allicin	Macaridin
	Ginkgolide J				MTCA
	bilobalide				
	Isorhamnetin				
	kaempferol				
	Quercetin				

These active ingredients are being analyzed using chemical methods (HPLC or LC-MS) in order to determine the real content of the chosen food supplements in these active ingredients.

Black radish and maca active ingredients have been analyzed so far.

The aim of the project is to determine the effects of these active ingredients on some physiological functions, using *in vitro* assays.

In order to perform these *in vitro* tests at realistic concentration in active ingredients, we calculated what we call "working concentrations", which correspond to plausible concentration of active ingredients at the human intestinal level, taking into account of the active ingredient content of FS and of the recommended daily intake of the FS.

So far, these realistic concentrations were calculated for St John's Wort, Gingko biloba and soy isoflavones.

3. Biological *in vitro* assays

The active ingredients (AI) were analyzed using several *in vitro* assays, in order to study :

- the general toxicity of the AI, on bacteria and eukaryotic cells (HepG2 and Caco2)
- the effect on targeted genes involved in oxidative damage, membrane damage, cellular stress and DNA damage
- the hormonal and dioxin-like activity using luciferase reporter gene assays
- the effect on human CYP1A and CYP3A4 activity in intestinal Caco2 cells

These analyses are ongoing and will be continued in the phase 2 of the project.

Detailed results are given in the text.

For example, we have evidenced that some AI of gingko biloba (flavonoids such as isorhamnetin, kaempferol and quercetin) are able to inhibit the dioxin activation of the Aryl Hydrocarbon receptor (Ahr), in hepatoma cells, and can also inhibit the BaP induced CYP1A1 activity in intestinal cells.

This kind of activity be may be considered as positive regarding detoxification of carcinogenic food contaminants such as PAHs.

Complete conclusions on the possible effects on physiological functions of active ingredients, at realistic concentrations, will be given at the end of phase II of the FOODINTER project, when a complete picture of all the *in vitro* effects measured with both pure standards of active ingredients and food supplements extracts will be available.

1. INTRODUCTION

1.1 Context

This project deals with an important aspect of the evolution of our relationships with food. We have moved to an increasingly complex food chain and consumer habits have dramatically changed. It is thus necessary to focus on the study of how new consumption habits evolve and on aspects of information and communication to the authorities, agro-food companies, health professionals and consumers.

Especially, food supplements and functional food become of increasingly great interest, as they are now consumed by more and more people These food supplements (e.g. nutrients, vitamins, hormones, amino acids, anti-oxidants,), as well as functional food (e.g. phytosterols or omega-3 fatty acids enriched food) occupy a position between food and drugs. Botanical materials represent a large segment of this class of products (e.g. soy isoflavones, yam or hop extracts). Para-pharmacy products, including phytotherapy products, (e.g. Chinese herbals, Sedinal, "tabac detox" with phyto-active substances, fish oil rich in omega-3 fatty acids, vitamins,) are also of major concern. The challenge ahead relates to quality, safety and efficacy. In many cases, there are still a number of unknowns such as the identification of specific active components and impurities, the effects of processing, the presence of toxic compounds, as well as their absorption and metabolism in the human body.

Our project includes research in areas such as innovative analytical protocols and their validation, quick detection methods and predictive *in vitro* models pertaining to chemical safety (endocrine disruptors, toxins, plant protection products, dioxins, hormones, polycyclic aromatic hydrocarbons - PAHs, …).

In the past, the attention of food toxicologists has been focused on the toxicity of single contaminating substances. The interactions between active and potentially toxic substances are poorly documented. Interactions can lead to additive or subtractive or even synergistic effects, which are being studied in FOODINTER.

This project aims to promote the communication between scientists and stakeholders (authorities, producers and consumers). In the field of food consumption, this objective is important because food safety depends not only on production and control, but also on consumption practices and good information must therefore be promoted. It is not only an education plan and the objective is also to promote a dialog between science and society in order to better identify the social preoccupations and need that research has to satisfy.

1.2 Objectives and expected outcomes

The objective of this project is to contribute to the risk assessment of chemicals, natural compounds and environmental contaminants, present in food supplements which could interact between them or with micro or macronutrients of normal human diet.

Interactions studies have been performed using existing *in vitro* models (based on culture of various cell types, prokaryotes and eukaryotes) with mixture of active substances at concentrations not yet studied until now and very close to the real situation in human nutrition. Extrapolation from the *in vitro* observations to the real risks for human will be attempted.

Our project will also analyze the place of food supplements in the diet and their impact on human health. It will increase the knowledge and fill some gaps regarding health claims and drawbacks that could be linked to these new habits in human nutrition.

Results will be communicated in order to increase the public confidence if risk assessment studies show no harmful effects on human health of new substances tested or to underline new risks, not taken into account previously. If necessary, recommendations will be formulated, in order to decrease emergent risks.

To reach the objectives, the following work packages are planned:

- WP 1. Preliminary information collection
- WP2. Biochemical and chemical analyses of contaminants, active substances of food supplements
- WP3. Risk assessment and communication, recommendations.

WP1 and a part of WP2 were performed in PHASE II. The second part of WP2 and WP3 will be achieved in PHASE II.

2. RESULTS AND DISCUSSION

2.1. Preliminary information collection

Within this task, several sources of information were consulted in order to gather basic information on food supplements but also in order to prepare the collection of samples on which the experimental studies will be performed. Different approaches were followed such as overview of the Belgian and European legislations, list of products notified in Belgium, overview on the information and choice of products available on the internet and in commercials, overview of the scientific literature dealing with health effects and interactions, contacts with producers and with consumers organized by means of surveys and focus group meetings, etc

2.1.1. Definitions according to current regulations, scientific literature and marketing practices

Food supplements, functional foods and para-pharmacy products are commonly used terms in nowadays scientific literature, regulation as well as advertisements. Nevertheless, acceptations regarding these terms are far from being shared by all, from consumers to stakeholders. For consumers, the situation may therefore be really confusing. It is of utter importance to establish precise definition to avoid miscomprehension and overlapping in product classifications.

The European Directive 2002/46/EC stated food supplements (FS) as "*foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities*". Examples of FS are ampoules of omega-3, tablets of vitamin A, tablets of multi-vitamin and multi-mineral capsules or capsules of plant extracts such as valerian, garlic,… Nowadays, there is a trend towards increasing marketing of plant based FS. Botanical material itself is not a FS. Example of botanical material are whole, fragmented or cut plants but also algae, fungi, or lichens are classified as botanicals. Botanical (or plant based) preparations can be obtained from these materials by various processes such as extraction, distillation, purification, concentration or fermentation (EFSA, 2004). Botanical preparations can be marketed either as medicinal products (see relevant EU and Member States legislations) or as FS. Since their introduction in the FS market, consumer exposure to some plant based preparations has become significant from a public health point of view. The present project will focus more precisely on the botanical preparations marketed as FS.

Functional food (FF) is a term created in the mid eighties in Japan after some researches on beneficial properties of foodstuffs. A functional food is similar in appearance to, or may be, a conventional food, is consumed as part of usual diet, and is demonstrated to have a physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions. Basically, there are two types of functional food: "FF inherently", i.e. food containing naturally beneficial components (omega-3 fatty acids in fish, flavonoids in fruits, lycopene in tomato,….) and "FF enriched", i.e. food enriched with beneficial components .(eggs enriched with omega-3, margarine with sterols, bread with polyphenols, juice with vitamins, milk with Ca, …). A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease. Example include capsules containing

bioflavonoids or gamma-linoleic acid. The term parapharmacy is indeed a widely used term in the field of human health. It encompasses products such as nutrients, FS, cosmetics, diet products, babyfood and several other products (surgical tapes, bandages,….).

2.1.2. European and Belgian regulations regarding food supplements

The Directive 2002/46/EC was adopted in 2002 in order to harmonize European regulations. Therefore this Directive helps gathering better conditions for food supplements free movement and equal competition conditions in Europe. Indeed, each country has its own regulation regarding FS, which is still in effect. The definition of FS in the Belgian regulation is very similar to the definition of FS in Directive 2002/46/EC (see 2.1.1). One can notice that the definition contains criteria regarding active substances and on their conditioning.

In Belgium, regulation of FS is grounded on two main Royal Decrees. The first one tackles the issue of Nutrients and their use into food supplements (AR 3/03/92). Among definitions stated in the documents, FS are defined as "*pre-dosed foodstuffs containing one or several nutrients, plants or plant preparations, or any other substance having a physiological or nutritive effect and which goal is to supplement normal diet*.", whereas nutrients are "*nutritive substances needed by the human organism*". Since the human organism is unable to produce these nutrients, adequate uptakes have to rely on foodstuff consumption. They are namely vitamins, minerals, amino acids, and fatty acids. Different dose forms in which dietary supplements can be available are also cited. This Royal Decree mentions the notifying process through which a FS has to go in order to be marketed in Belgium. Maximal and minimal limits in terms of % of the Dietary Reference Intake (DRI) are fixed for different nutrients used in FS. Guidelines are detailing the labelling and the advertising of these FS. The DRI for vitamins and minerals, foodstuff consumption data and forbidden product are cited in three annexes accompanying the Decree. The second Royal Decree concerns plants and plant preparations (AR 29/08/1997). It contains a list of dangerous plants whose use for direct consumption or as ingredient of preparation is strictly prohibited. Besides, another list gathers all plants allowed for direct consumption or as ingredient of preparation as long as a notification file has been accepted by federal authorities.

2.1.3. Marketing and consumption of food supplements

Throughout the world, sales of FS have grown dramatically over the last decade. In 2000, world sales were reaching billions dollars whose 37% in the USA, 30% in Europe and 28% in Japan. The two most important FS families sold are plants or plant extracts (42%) and nutrients (39%). FS are sold on the market through several paths, mainly through pharmacies (drugstores), specialized stores (dietetic shop, organic shop, beauty shop,…), retailers, internet sales and direct mail orders. For different reasons, sales in Belgium are difficult to estimate because data from retailers and specialized stores are difficult to obtain. In drugstores, FS sales data are stored under the item "parapharmacy product" leading to a consequent overestimation. In addition, sales done on internet or by mail orders are slipping through any controls. To provide a gross estimation it is said that the Belgian market is equal to 1/5-1/6 of the French market or in other words about 150 millions € were spent in 2004 in Belgium.

The Food Consumption Study performed by the Scientific Institute of Public Health in 2004 provides us data on FS consumption within the Belgian population (De Vriese et al., 2006). The study showed that 11.5 % of the population has included FS in their diet. Regarding to the gender issue, 13.1 % of women are consuming FS whereas for men this percentage reaches 9.8

%. FS consumption increases with age, as the highest percentage (16 %) of consumption is reached for the age group 60-74 years old. Regional tendencies can be noticed since dietary supplements are consumed for 12.8 % of the people interviewed in the Flemish Region, while for the Walloon Region and the Brussels Region this figure is reaching respectively 10.1 % and 8.4 %. In terms of FS composition, the highest consumption percentage concerns vitamin/mineral cocktails followed by vitamins, minerals, plant extracts, and others. Another source of national data is given by Huybrechts and De Henauw (2007). In this study, set up to investigate the energy and nutrients intakes of pre-school children, daily diet of 661 children in the Flemish Region was assessed in 2002-2003. Data gathered showed that 32 % of these children (between 2.5 and 6.5 years old) were consuming FS made of vitamins and minerals

2.1.4. Overview on the available information about health incidents, contaminations and interactive effects reported in the literature and related to botanical preparations and food supplements.

Herbal preparations originated from Asia did cause problems through contamination with heavy metals, synthetic drugs and other substances (Ernst, 2002). Misidentification of plants harvested in the wild is sometimes source of major poisoning. Particular concerns are coming from plant products consumed in concentrated forms. Since acute liver failing was associated with kava-kava (*Piper methysticum*) consumption, this product has been withdrawn form the market. In June 2001, the US FDA advised manufacturers of FS to avoid the use of the various types of comfrey (*Symphytum spp*.) because of the health concerns associated with the presence of pyrrolizidine alkaloids (EFSA, 2004). Moreover, since 1993, cases of nephrotoxicity and carcinogenicity have been reported in Belgium, France and United Kingdom as a result of inadvertent exposure to *Aristolochia* species in unlicensed herbal medicines (EMEA, 2000). Another concern was raised after various intoxications subsequent to star anise consumption. Trees from which are issued both star anises are very similar, causing in the past a lot of contamination of Chinese star anise by the Japanese star anise in herbal teas. Chinese star anise helps for the treatments of colicky pain in infant, whereas Japanese star anise can provoke tremors or spasms, hypertonia, hyperexcitability with crying, nystagmus, and vomiting (Minodier et al., 2003). The estragole, contained in star anise and fennel, was also pointed out for its carcinogenicity by the European Medecines Agency. Even if exposure to estragole through herbal medicines was considered as not significantly risky, concerns were brought concerning the exposure for sensitive groups such as toddlers, pregnant women and breastfeeding women. Several risks are encountered when herbal medicines are consumed: wrong identification of plants due to plant similarity or contamination of cultivated plants by weeds, contamination by environmental contaminants (heavy metals, …), intrinsic toxicity resulting from the presence of natural toxins within plants and interactions with drugs or contaminants found in food diet.

Interactive effects may occur between plant active substances and other components such as food contaminants and drugs. Contaminants in food are heavy metals, polycyclic aromatic hydrocarbon (PAH), dioxins, mycotoxins, pesticides, polychlorinated biphenyls (PCBs), … but those chemicals can also be present as natural, environmental and process contaminants in the FS itself. Literature detailing interactive effects between contaminants and active substances is quite poor. Nevertheless, interactive effects often occur at the level of enzymatic activity (Sergent et al., 2008). Thus, competition or inhibition of enzyme activity can lead to accumulation of toxic substances. Interactions of herbal products with prescription products are well recognised, although widely under-reported (Ernst, 2002).

In 2006 and 2007, the FASFC (Belgian Food Agency) published two warning concerning dioxin concentration exceeding norms into fish oil capsules. In 2006, two persons were intoxicated in Belgium after ingestion of capsules containing herbal preparations. Products analysis revealed a high level of lead concentration. In this case, capsules were distributed as therapeutic products by an Indian sorcerer. Another problem occurred in slimming pills sold by a Belgian retailer. Arsenic concentrations were indeed exceeding Belgian norm.

2.1.5. Information gathered via surveys and focus groups

Consumer's standpoints about food supplements were collected by the teams of ULg (Socio Economy Environment and Development, Marc Mormont and co-workers) and CERVA-CODA (Luc Pussemier and co-workers) using a questionnaire presented in Annex 1. Another survey, not initially planned in the project, was performed in Flanders by students of the Faculty of pharmacy of the University of Ghent, under the supervision of Sarah Desaeger and Carlos Van Peteghem. The results of this last survey have been structured in a table (see annex 2). Nevertheless, they are not yet interpreted and the conclusions will not be presented here.

Functional foods and food supplements are a challenge to food health policies. Sociological literature concerning food trends show that technical rationality does not fully explain consumer's attitudes and choices, and that other food related rationalities, such as practical and economic rationalities, social and relational rationalities, and symbolic rationalities do play a role in eating habits. An exploratory analysis of lay views shows that scepticism co-exists with interest in food supplements and functional food. Ambiguity characterizes consumer's representations and practices.

The objectives of this study focus on food supplements that are well known by many consumers and that are taking a larger and larger place in consumption? As a first objective, we tried to characterize opinions and representations of consumers and non consumers. As a second objective, we tried to explore more in details these representations by confronting a group of consumers to expert knowledge (science and legal specialists, but also a producer), and as a third objective, we interviewed a few producers to question the way they define consumption and related risks.

Methodology : The survey by questionnaire was intended for clients of supermarkets, food stores, drugstores and specialized (organic) food stores from Brussel and Liège, two important Belgian cities. In addition, the same questionnaire has been used for interviews carried out by undergraduate pharmacists (apotheker-stagiair) into drugstores of the Northern part of Belgium. The questionnaire (see annex 1) consisted of 20 questions (both open and closed questions asked in face-to-dace meetings) related to: i) the perception of food supplements and functional foods, ii) the frequency of their consumption, iii) the budget allocated to their consumption, and iv) the perception of possible risks. For Brussel and Liège, 167 survey questionnaires were distributed and answered (74% of the interviewees were FS consumers). For the survey in the drugstores of Northern Belgium the number of answered questionnaires was 277. Nevertheless, the conclusions given below do not yet include the results of this last survey among Flemish consumers.

Social representations of food supplements were examined with a focus group methodology.

The Focus Group survey was intended for both consumers and non-consumers, carried out in three meetings of two hours each. The number of participants varied between 6 and 12. These three meetings permitted the participants to discuss food supplements and functional foods. Four outside participants also contributed as experts to these discussions through presentations. This

group was heterogeneous in terms of age, social situation but most of the participants were woman more or less interested in the question. The first meeting was intended to give them basic scientific information and to identify points to be explored and discussed. The second meeting allowed the participants to acquire information and the legal and administrative aspects and to receive information from a producer. The last meeting consisted in an open and extensive discussion and was intended to formulate some proposals for policy-making. Within both the interviewees and within the focus group participants, working class strata were under represented probably for cultural reasons.

Finally semi-directive interviews were held of representatives of food supplement producers. Four different producers were interviewed to explore the way producers manage the risk aspects of food in this specific context. Two of them were active in the "custom" trade. An interview guide has been compiled up to serve as a framework for the interview, while the interview consisted principally of inquiring into their traceability systems, contaminant and interaction-related risk management systems, their opinions on product regulation, etc..

Results : From a first analysis of the results, it appeared that : 1) people do not exactly know what kind of preparations can be categorized as food supplements (a lot of hesitation for vitamins and plant extracts), 2) women seem to consume food supplements more often than men, 3) 37% of questioned people do consume food supplements from their own initiative (without medical advice) , 4) the main purpose of consuming food supplements is, according to consumers, to reinforce the immune system of the organism and to fight against tiredness (obviously for vitamins and mineral), 5) a lot of consumers are regular customers but mostly the money spend for buying food supplements is less than 50 Euros, 6) most of the consumers do read the label and are convinced of the beneficial effects as they are described in the label, 7) the majority of the questioned people do believe that food supplements are "natural" but seem to be aware that simultaneous intake of drugs can pose a health risk.

From interviews in the sales places, functional food and food supplements are not fully understood by consumers, but it is not ignorance at all. In general most of the consumers adequately distinguish food, medicine and functional food or supplements. And knowledge is better when consumption is intensive or regular. Then it can be concluded that consumers are looking for information: actually they all read information if given by producers. More than one third of the consumers were given advices by doctors. One on four use supplements for preventive reason but the great majority consumes them for reason linked to chronic (real or supposed) deficiencies, for stress and tiredness. Most of them concede some kind of risks in this consumption but declare to make adequate use of them. These results, among others, confirm that consumption is not irrational and that it is information driven. So the role of information by physicians or by other sources can play a crucial role. Most of them do not entirely trust either medicine or food supplements, but consumption can be related to some representation of nature since these products seem quite natural to them. It can be noticed that most of them seem very cautious regarding food and health, probably more than non consumers on the average. There is a sort of ambiguity in these attitudes since consumers are at the same time interested in natural and well-balanced diet and users of these products.

During the focus group sessions, the problem of obtaining sufficient information was frequently raised, in a variety of different forms; the problems related not only to publicity but to the presence, absence and content of the notices either enclosed within the packages or printed on the package itself, as well as the patient/physician dialogue. The topic of economic interests, strongly linked to that of advertising and trust in members of the healthy industry, quickly made its appearance during the first meeting, and then became a recurrent topic throughout the following meetings as well. Thus, perception of the larges-scale producers of food producers is clearly negative. The reasons for this poor image are, overall, said to be related to the notion that

they are primarily seeking to make money, particularly, through advertising. In addition, according to the participants, food supplements which base their claim to legitimacy on the nutritional imbalance of our societies, does not encourage nutritional balance, but maintains the imbalance. The demand for stricter regulation has been made very frequently. Despite the information provided on the work carried out by the Federal Public Service on Public Health, several critical remarks were made about the public authorities. First of all, three criticisms were expressed with regards to the certification procedure (small number of persons responsible for analysing the thousands of certification applications). Another criticism related to the absence of the certification number on the packages, as a quality control guarantee. The third criticism related to the possibility, for producers, of placing products on the market which had not been notified. Finally, the absence of clarity in the regulations relating to "health claims" was underlined. Finally, the participants asked for more complete and accurate information on the packages and on the presence and contents of the labels. It should be noted that there was also a demand for compulsory information on the proven effectiveness of the products. As to the consumer's perception regarding FS, it can be noticed that for some people, food supplements are a vital health care necessity and remedy for deficiencies whilst, for others, food supplements are well-being products which are not physiologically vital but important to people in their quest for good health and well-being.

The focus group methodology allowed consumers to explore more in depth and to discuss different aspects. First it appeared that consumption is not naïve for most of them. It also indicates that individual attitudes are very diverse and deeply rooted in individual experience with health problems. Discussion between participants reveals that there is no contradiction between natural food and balanced diet (what they consider the ideal) and consumption of supplements since for them many people have health problems that can be alleviated by food supplements.

For most of them it is a reflexive practice. Consumers do not trust the commercial system to provide good products and they ask for more information from producers and form public authorities. They do not feel at risk but they regret what they perceive as weaknesses in the control. Concerning the research project (Foodinter) they feel dubious about the expected results of laboratory research and ask for a good communication of these results to the public. In general they trust scientists to improve this knowledge.

Interviews of producers revealed a very cautious attitude concerning traceability and quality.

Conclusion : From these results we can conclude on a hypothetical way that food supplements, even if consumption is growing, reveal that consumers do not entirely either trust commercial food or medicine. Food supplements are rather clearly distinguished from drugs and from food. As far as consumers of supplements are concerned, they are suspicious and they try, with a good reflexivity, to find solutions to chronic health problems that seem to be linked with their way of life. They consider supplements as improvements, keeping in mind a good idea of well balanced diet. Information and better control are the main preoccupations they formulate, with an emphasis on independence of control, of research and of public information.

2.2 Collection of samples and construction of a database

Samples have been collected to perform the studies of interactive effects with food constituents, chemical contaminants and other relevant chemicals (medicinal products). For this project, special attention was drawn to FS made from botanical preparations because their increasing use as FS and their relatively high contents in biologically active ingredients make them more prone to contribute significantly to the consumers' exposure.

2.2.1. Selection of food supplements to be studied in detail and collection of the Samples

The selection of FS for further studies was carried out by taking into consideration several criteria which are:

1) Active ingredients potentially susceptible to interact with some key enzymatic systems such as those involved in phase 1 en 2 metabolism. To meet this criterion it was obvious that several plant based products will rank high due to the nature of their biologically active ingredients.

2) Frequently used products (i.e. distributed by many producers, mentioned by consumers during the surveys or mentioned in many commercial advertisements)

3) Products more prone to be contaminated by several kind of contaminants (plant toxins, mycotoxins, heavy metals, PAH, dioxins and PCB). To meet this criterion, plant based products obtained according to extensive cultivation methods will rank high (exposure to environmental pollution, non professional drying and extraction processes, etc).

4) Exotic products susceptible to contain active ingredients that are less studied up to now (emerging risk potential)

Despite the fact that vitamin and mineral based FS are the most frequently used according the information gathered (see above), they were discarded from the selection because, in the framework of this project, the potential interactive effects with key enzyme systems were considered as a top priority for selection. The final selection was made of six different FS all made from one specific plant material (no mixtures!) and complying with criterion 1. In addition four of them were in accordance with criterion 2. Two others FS were added despite the fact that they are not frequently used but because they responded to criteria 3 and/or 4. The results of the final selection are presented in table 1.

Table 1 : Final selection of food supplements[1] to be studied within the FOODINTER project

FS	Uses/biological effects	Criteria for selection	number of samples collected	
			Notified in Be	Not notified in Be
Ginkgo biloba (B)	Improve blood circulation and cerebral oxygenation	# 1, 2, 3	5	7
Sint-John's wort (W)	Mild depression	# 1, 2, 3	5	8
Soy isoflavones (I)	Reduce menopause effects	# 1, 2	5	7
Garlic (G)	Decrease arterial tension	# 1	5	7
Maca (M)	Increase libido and limit sexual disorders	# 1, 3, 4	3	3
Black radish ®	Bile secretion stimulation and intestine activity	# 1, 3	2	4

[1] Not notified products are not food supplements (in its strictly legal meaning) but just food products

In total, 61 samples were thus collected (see Annex 3). They were purchased from 37 companies via internet (36 samples), drugstores (18 samples) and specialized shops (7 samples). 25 are notified in Belgium whilst 36 are not notified (and generally available via the internet). 43 FS were obtained as capsules containing solid plant material or extract, 13 FS were tablets and 6 were available as oily capsules. This material has been dispatched to the different labs in order to perform the analyses of chemical contaminants, of active ingredients as well as for the *in vitro* studies.
A data base has been compiled using all the relevant information on the uses of FS, the nature of their active ingredients, the contents of active ingredient s in the FS, the intake of active ingredient according to the recommended doses, the methods of analysis, the biological studies performed on the active ingredients, etc

2.2.2. Analysis of chemical contaminants

2.2.2.1. Analysis of mineral elements

Methodology. Preparation of samples to be analyzed consisted in grinding 5 units from the dietary supplements in order to obtain a homogenized material. For capsules and oils, the content was released from the packing and carefully mixed. The tablets were crushed and mixed. 17 trace elements (As, Ba, Bi, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Se, Sr, Ti, Tl, Zn) were quantified by inductively coupled plasma with mass spectrometer (ICP-MS). Mercury (Hg) was quantified by Advanced Mercury Analyzer (AMA).For ICP-MS measurement, 0,25g of each homogenized sample was taken and mineralized under microwave heating. This digestion was performed at 180°C under temperature and pressure control in presence of concentrated nitric acid. After cooling, the digests were diluted 400 times prior to analysis, to avoid any matrix effect. As Mercury shows memory effects with ICP-MS, this element was quantified by the means of an Advanced Mercury Analyzer (AMA). This technique, specific for mercury, needs no digestion, as analyze is directly performed on non-treated sample. About 100 mg of sample is weighted and introduced in the instrument where it undergoes the following steps : burning, amalgamating with gold, evaporating and detection by atomic absorption spectroscopy. Each sample was analyzed twice. To assess quality control, reference materials from International Atomic Energy Agency (IAEA 155, certified milk powder and IAEA 407, certified fish tissue) were analyzed by following the same preparation as the samples. Blanks were analyzed before each batch of 20 samples as well as a standard solution of 10 µg/L to correct any drift effect. The limit of quantification (LOQ) was determined as 10 times the standard deviation of 10 blanks multiplied by the dilution factor. No significant variation was observed between the 2 replicates for each sample tested, which means that samples were correctly homogenized. All calculations were done using the mean value of the two replicates. The sample which shows a lower value than LOQ were equalled to LOQ/2 for further treatment of the results

Results. The results obtained for the 18 elements in 62 samples (one of the 61 samples contained 2 different types of capsules) are summarized in table 2.

Table 2 : Summary statistics for 18 elements measured in the 61 FS of the sample bank.

Element	Units	Mean	Median	Standard Deviation	Minimum	Maximum	Lower Quartile	Upper Quartile
Hg	ppb	7,21	1,55	15,83	0,09	86,10	0,46	4,04
Ti	ppb	4311,87	1938,00	8197,16	140,00	49755,00	436,85	3629,50
V	ppb	611,36	187,30	1326,62	35,00	8926,00	93,91	628,95
Cr	ppb	2413,00	825,35	9653,52	100,00	75810,00	398,40	1493,00
Mn	ppm	67,65	12,01	399,89	0,02	3136,50	4,16	25,45
Co	ppb	302,70	92,34	620,64	2,50	4622,00	24,74	406,90
Ni	ppb	1921,31	1079,00	2751,57	25,00	15370,00	520,80	2238,00
Cu	ppm	10,45	3,15	27,63	0,02	159,55	1,45	7,65
Zn	ppm	285,14	17,60	1486,57	0,45	9725,50	8,06	28,67
As	ppb	152,73	90,40	170,28	15,00	900,80	37,58	236,40
Se	ppm	5,39	0,05	26,48	0,05	197,60	0,05	0,18
Sr	ppm	10,29	7,08	10,95	0,05	45,99	1,39	14,77
Mo	ppb	632,64	319,10	1090,40	40,00	6436,00	90,05	556,45
Cd	ppb	134,11	47,96	224,01	1,00	995,75	9,15	166,35
Ba	ppm	6,76	1,75	11,76	0,02	80,72	0,74	9,58
Tl	ppb	17,35	4,36	25,74	1,00	118,00	1,50	19,55
Pb	ppb	1893,07	130,95	9558,49	3,00	73870,00	33,39	508,55
Bi	ppb	15,62	12,50	13,52	12,50	99,24	12,50	12,50

Discussion. It is important to note that there were 10 non compliant (NC) samples with respect to the Belgian legislation for toxic element in FS (7 NC for Pb and 4 NC for Cd; one sample exceeded the norm for both elements – see table 3).

Table 3 : Non compliant (NC) samples
(M = Maca ; B = Ginkgo biloba ; W = St John's wort)

Belgian maximal limit (ppb)	Type	Hg	As	Cd	Pb	Notified
		200	1000	500	1000	
Active maca	M	-	-	931	-	N
Ail	B	-	-	-	2314	N
Gingko biloba 400	B	-	-	-	6707	Y
Ginkgo biloba	B	-	-	-	3202	N
Maca	M	-	-	552	-	Y
Maca	M	-	-	996	-	N
Millepertuis	W	-	-	958	73870	N
Millepertuis	W	-	-	-	1412	Y
Millepertuis Fort	W	-	-	-	13020	Y
St John's Wort 60	W	-	-	-	2107	N

It has to be stressed that the exceeding of the maximal limit was in one particular case very dramatic (74 fold exceeding of the Pb norm for a St John's wort based FS) and that the Belgian Food Agency has been informed about the NC samples according to the Belgian regulations.

2.2.2.2. Analysis of mycotoxins

The aim of this study was to provide an analytical methodology for the simultaneous determination of different mycotoxins in food supplements and to further apply this methodology for the analysis of the food supplements selected in the framework of this project. The methodology was based on a previously developed liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of 16 mycotoxins in fungal cultures (Delmulle *et al.*, 2006). The LC-MS/MS conditions were adapted to encompass 23 mycotoxins and an extraction procedure as well as a sample clean-up methodology were optimized for food supplements.

Materials and methods : Experiments described here were performed using gradient reversed-phase liquid chromatography (RP-LC) with electrospray ionization tandem mass spectrometry (ESI-MS/MS). Detection of the mycotoxins was carried out in the multiple reaction monitoring (MRM) mode. Mycotoxins were extracted using acidified ethyl acetate (ethyl acetate / formic acid (95/5, *v/v*)) as extraction solvent. Sample clean-up involved an n-hexane defatting step followed by a solid phase extraction (SPE) step on an Oasis SPE cartridge. The different samples were screened for the presence of 23 mycotoxins (See below). For positive samples, the mycotoxins were quantified by the standard addition approach.

Results and discussion : The target mycotoxins included nivalenol (NIV), deoxynivalenol (DON), neosolaniol (NEO), fusarenon-X (F-X), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), T-2 toxin (T-2), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA), altenuen (ALT), alternariol (AOH), alternariol methylether (AME), fumonisin B1 (FB1), fumonisin B2 (FB2), fumonisin B3 (FB3), zearalenon (ZEA), beauvericin (BEAU), sterigmatocystin (STERIG). In 54 out of 61 food supplements analyzed none of the 23 mycotoxins was detected. The toxins FB1, FB2, FB3 and OTA were detected in some samples (Table 4). In 2 samples, OTA was found at a level above 2 µg/kg (EC norm for wine and grape juice, Regulation 1881/2006/EC). The levels of FB1, FB2 and FB3 were largely below 800 µg/kg (EC norm for the sum of FB1 and FB2 in breakfast cereals, Regulation 1881/2006/EC) in all samples.

Table 4. Mycotoxin contamination (ppb) in food supplements

Product	*CODE FS*	*CERVA N°*	*Notified*	*Origin*	*FB1*	*FB2*	*FB3*	*OTA*
Kyolic	G	22	Y	S	<1	<0,3	<1	6
MACA 500	M	29	Y	D	<1	<0,3	<1	2,5
Elusan Ail	G	36	Y	D	10	8	3	1
Isoflavones 50	I	41	Y	D	4	<1	<1	<0,3
Radis noir	R	53	N	I	4	2	<3	<0,3
Meno (jour)	I	62a	N	I	<1	<0,3	<1	1
Meno (nuit)	I	62b	N	I	<1	<0,3	<1	1

2.2.2.3. Analysis of PAHs

Polycyclic aromatic hydrocarbons (PAHs) are a large group of more than 200 different chemicals containing two or more fused aromatic rings made up of carbon and hydrogen atoms (NIST). PAHs are formed during the incomplete burning of organic substances and pyrolysis processes (Mc Grath et al, 2001, Wang et al, 2001). They are widely present in the environment due to their lipophilic properties allowing their adsorption on the atmospheric particles and deposition in sediments, soils and plants.

In the European Union, the Scientific Committee on Food (today EFSA) published in 2002 a list of 15 priority PAHs for controlling PAHs contamination in food (SCF, 2002) . In 2005, the Joint FAO/WHO Experts Committee on Food Additives (JECFA) appended to the list a 16^{th} compound (benzo[c]fluorene, BcL) also considered as genotoxic (FAO/WHO, 2005).

Maximum tolerable levels exist for benzo[a]pyrene (BaP) in smoke condensates (Regulation 2065/2003/EC) and in diverse foodstuff categories (Regulation 1881/2006/EC). Until now BaP is used as a marker for the occurrence of the carcinogenic PAHs.

Food supplements may contain large contaminations with PAHs (FSA, 2005) due to the environment and/or production practices, such as the drying step before the grinding of the plant. The risk associated with PAHs in food supplements was recognized by the Commission but further investigation is needed to fix maximum tolerable levels. There is currently no legislation in place controlling the maximum levels of BaP or other PAHs in food supplements. In the mean time, maximum levels for BaP in oils and fats are applied; they are fixed to 2 $\mu g\ kg^{-1}$.

High performance liquid chromatography coupled to an ultraviolet, diode array or fluorescence detector (HPLC/UV-FLD) (Brasseur et al, 2007) has been used to detect the 15(+1) EU priority PAHs in the sixty food supplements selected in this project.

Results obtained for the 16 PAHs are presented in detail in annex 4. The results have shown that S^t-John's wort and ginkgo biloba extracts presented the most frequent contaminations and the highest average values for PAHs concentrations.. The most contaminated samples with the sum of the 16 PAHs were generally detected in St-John's wort and ginkgo products, except one sample of soy isoflavones . The most frequent PAHs detected were benz[a]anthracene (BaA) and benzo[a]pyrene (BaP) in all the types of plants tested, chrysene (CHR) was rather frequent too . From the results obtained 29 samples, on a total of 60 tested, showed a concentration higher than 2 ppb for one or more EU priority PAHs concentrations, and only 9 of these contaminated samples were notified. Generally, not notified products bought by internet showed higher contaminations.

In order to perform a preliminary risk assessment, we calculated the daily intake of the sum of the 16 PAHs from food supplement taking into account of the PAHs concentrations and the recommended ingestion per day of each analyzed food supplement (see annex 5). Because a number of PAHs have been shown to be genotoxic carcinogens, no acceptable daily intake (ADI) exist.

To evaluate the risk coming from food supplement ingestion, we propose to use 3 different approaches of the literature.

First, we compared the PAH intake from food supplement with the tolerable maximal daily intake (TMDI) calculated for BaP in the EMRISK project (Ribonnet *et al*., 2007). The TMDI of BaP was calculated by multiplying consumption data from GEMS-FOOD regional diets (2003) by the maximum levels fixed for BaP in food in the European Regulation (RE

1881/2006). If the PAH intake from food supplement is higher than the TMDI (= 273 ng/day), we assume a human risk from intake of PAH from food supplement.

The second approach is the principle of the margin of exposure (MOE) used by the JECFA (2005). We calculated the MOE by dividing the toxic PAH intake (BaP + 13genotoxic carcinogens PAHs) determined from animal experiments by the PAH intake from food supplements. This toxic intake is called benchmark dose lower confidence limit (BMDL) and has been estimated to 100 µg/kg bw/day in mice (oral administration). Consequently, the lower is the MOE (which represents a kind of safety ratio), the greater is the public health concern. In 2005, JECFA performed a risk assessment for PAHs and, on the basis of a human mean intake of 4 ng/kg bw/day, and a high-level intake of 10 ng/ kg bw/day, estimated a MOE of 25.000 and 10.000 respectively. They concluded that estimated intake of PAHs from food was of low concern for human health. Consequently, in this work, we considered that if MOE is higher than 10000, there is no human health concern regarding ingestion of PAHs from food supplements.

In the third approach, from the RIVM, we used a tolerable intake level of 5 ng BaP/kg/day determined by Baars *et al.,* 2001, by applying the linear non-threshold approach. Assuming a body weight of 70 kg, we calculated a virtual dose sure (VDS) of 350 ng/day. If the PAH intake from food supplements is higher than this VDS, we assume a human health concern.

With the first and third approaches, we found five and four food supplements of health concern, respectively. Samples 45 (Black radish), 55 (Ginkgo biloba), 58 and 67 (both St John's Wort) are of human health concern with both approaches. Sample 51 (Garlic) is of health concern according to the fist approach only. All are not notified. By using the principle of the JECFA approach, we found no food supplement of health concern.

2.2.2.4. Analysis of organochlorine pesticides (OCPs), polychlorinated biphenyls(PCBs), polybrominated diphenyl ethers (PBDEs) and dioxin in oily FS

For the analysis of OCPs, PCBs and PBDEs in the food samples only the oily samples were selected for analysis.

The analytical method for the simultaneous determination of various groups of persistent organic pollutants (organochlorine pesticides, polychlorinated biphenyls and polybrominated diphenyl ethers) was previously described by Jacobs et al., (2004). This method was applied for the analysis of the food supplements selected in the framework of this project.

Materials and methods: Experiments described here were performed using gas chromatography with mass spectrometry (GC-MS). Detection of the each individual pollutant was carried out using specific ions in well-defined windows of the chromatogram. An exact aliquot of ~0.15 g of food supplement was solubilized in 3 mL of *n*-hexane, internal standards were added, and the mixture was equilibrated in an ultrasonic bath for 5 min. The extract was applied to an *n*-hexane prewashed cartridge filled with 8 g of acidified silica (44%, w/w) and was eluted with 15 mL of *n*-hexane and 10 mL of dichloromethane. The final eluate was concentrated with a rotary evaporator and further under nitrogen and the dried residue was reconstituted in *iso*-octane. Method limits of determination for individual compounds were 0.2 ng/g for individual PBDEs and 0.5 ng/g for individual OCPs and PCBs. Quality Assurance was ensured through analyses of procedural blanks and certified material CRM 350 (PCBs and organochlorine pesticides in mackerel oil).

Results : The results (table 5) show low amounts of p,p'-DDE present in 3 samples (Garlic Vitaal Forte, Max Gar Garlic and Ail Special eXtra) and "Ail Special eXtra" also contains

low levels of o,p'-DDD. The allowed levels for the sum of DDT is 50 ng/g garlic, none of the food supplement were above that level (Regulation 396/2005/EC.). None of the OCPs and PBDEs were detected above the limit of quantification.

Table 5: OCPs, PCBs and PBDEs in oily food supplements. HCH – hexachlorocyclohexane; HCB – hexachlorobenzene. For calculations, values below limit of quantification were replaced with ½*LOQ.

	Concentration (ng/g oil)					
	Life extension PC Ginkgo biloba	**Garlic Vitaal Forte**	**Max Gar Garlic**	**Ail Special eXtra**	**Bakanasan Huile de Milepertuis**	**Ail**
Mass oil (g)	0.13	0.15	0.14	0.13	0.15	0.14
α-HCH	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
β-HCH	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
γ-HCH	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Sum HCHs	**< 0.8**	**< 0.8**	**< 0.8**	**< 0.8**	**< 0.8**	**< 0.8**
Trans-chlordane	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
Cis-chlordane	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
Oxychlordane	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
Trans-nonachlor	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
Sum Chlordanes	**< 0.6**	**< 0.6**	**< 0.6**	**< 0.6**	**< 0.6**	**< 0.6**
HCB	**< 0.5**	**< 0.5**	**< 0.5**	**< 0.5**	**< 0.5**	**< 0.5**
p,p'-DDE	< 0.5	6.0	1.2	1.9	< 0.5	< 0.5
o,p'-DDD	< 0.5	< 0.5	< 0.5	1.7	< 0.5	< 0.5
p,p'-DDD	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
o,p'-DDT	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
p,p'-DDT	< 0.5	2.0	< 0.5	< 0.5	< 0.5	< 0.5
Sum DDTs	**< 1.3**	**8.8**	**2.2**	**4.2**	**< 1.3**	**< 1.3**
PCB 28	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PCB 52	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PCB 101	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PCB 118	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PCB 138	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PCB 153	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PCB 180	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Sum 7 marker PCBs	**< 1.8**	**< 1.8**	**< 1.8**	**< 1.8**	**< 1.8**	**< 1.8**
PCB 105	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PCB 156	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PCB 170	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
PBDE 28	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
PBDE 47	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
PBDE 99	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
PBDE 100	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
PBDE 153	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
PBDE 154	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
PBDE 183	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
Sum PBDEs	**< 0.7**	**< 0.7**	**< 0.7**	**< 0.7**	**< 0.7**	**< 0.7**

Dioxins were measured in these oily samples using the CALUX method (Scippo et al, 2004). Because of the low amount of remaining food supplement (only 1 g), the limit of quantification (LOQ) of the method was of 2 pg TEQ/g oil which is a little bit higher than the maximal level of 1.5 pg TEQ / vegetable oil for hman consumption (Regulation 1881/2006/EC). All the samples were below the LOQ.

2.2.2.5. Constitution of a data base for statistical analysis

Methodology. It is the aim to build a common database with all the results obtained from analytical measurements performed on the sample collection gathered with the FOODINTER project (i.e. 61 samples). This data base will provide, under a standardized format (Excell worksheets), all the relevant information needed for a thorough analysis of the results according to uniform procedures for data handling. The database will contain for each of the measured parameters (trace elements, mycotoxins, PAH, alkaloids, glucosinolates, …) the following information on the analysed samples: Code nr of the sample, type of FS (Garlic, Ginkgo biloba, Soy isoflavones, etc), type of formulation (capsules, tablets, oily gelules), brand name, producer, information on notification in Belgium (notified or not), Limit of quantification, norm to be applied according to Belgian legislation, etc.

Results. An example of the used format and of the information provided is given in table 6.

Table 6 : Format of the database under construction for the handling of results gathered on 61 FS samples within the FOODINTER project.

						Analyte	**Hg**	**Ti**	**V**
						units	ppb	ppb	ppb
						LOQ	**0,17**	**280**	**70**
						BE Norm	**200**		
CODE FS	**CERVA N°**	**Notification**	**Origin**	**preparation**	**producer**	**Name**			
W	**1**	**Y**	**I**	**C**	PARABOLIC BIOLOGICAL S	SINT-JANSKRUID STERK	1,55	<280	148
B	**2**	**N**	**I**	**C**	ORTHICA	GINKGO 90 capsules	3,26	1319	121
B	**3**	**N**	**I**	**C**	BLOEM	ginkgo extra forte bloem	86,1	4596	708
B	**4**	**N**	**I**	**O**	LIBERTY HEALTHCARE	Life extension PC Ginkgo	0,365	<280	<70
W	**5**	**Y**	**I**	**T**	LICHTWER PHARMA BENE	Kira® Sint Janskruid LI 160	<0,17	407	115
W	**6**	**N**	**I**	**C**	SPRINGFIELD	ST JANSKRUID 500MG	1,61	3358	207

CODE FS	Classification per type of **D**ietary **S**upplement (**B** = Gingko **B**iloba, **G** = **G**arlic, **I** = soy **I**soflavone, **M** = **M**aca, **R** = black **R**adish, **W** = St John's **W**ort)
CERVA Nr	First number given by CERVA when bought in shops or via internet
Analyte	Chemical analysed (trace element, mycotoxin, PAH, etc)
Units	ppm = microg/g dry weight; ppb = ng/ g dry weight
LOQ	Limit of Quantifification
Be norm	Legal norm in Be (max concentration)
Notification	Y = Notified in Belgium; N = Not notified in Belgium
origin	D = Drugstore (pharmacy), S = specialized Shop, I = internet
preparation	C = Capsules (or gelules), T = Tablets, O = Oil capsules
Name	Brand name of the food supplement
Producer	Identification of the producer or distributor

The whole database (still in construction !) is presented in annex 6.

2.2.3. Identification of active ingredients

2.2.3.1. Identification of active ingredients from literature

1. Soy isoflavones. The products of this group contain different isoflavones of soy extracts as active compounds. Indeed, beside the nutritional benefits of soy proteins, the interest in soy foods is also driven by the fact that they offer a suitable delivery system for attaining high plasma concentrations of isoflavones (Zubik *et al.*, 2003). As a consequence, many extracts of isoflavones have become widely available as food supplements (Messina *et al*., 2003). The parent isoflavones are aglycone structures of daidzein, genistein and glycitein, which are conjugated to malonyl, acetyl and β-glucosides moieties. The hormonal activity of soy isoflavone products can be attributed to their similarity in structure with the mammalian oestrogen 17-β-oestradiol (Setchell et al., 1999). For the soy isoflavone products under investigation in this project, the total amount of isoflavones contribute for 40 % of the soy extracts.

2. Maca. The products of this group are based on maca (*Lepidium meyenii*) extracts. They are claimed to increase stamina and fertility. It is believed that maca's beneficial effects on sexual performances can simply be explained by its high concentration of proteins and vital nutrients. However, maca contains a chemical called p-methoxybenzyl isothiocyanate, which has reputed aphrodisiac properties. The methanol extract of maca tuber also contains (1R,3S)-1-methyltetrahydro-carboline-3-carboxylic acid, a molecule which is reported to exert many activities on the central nervous system. In addition to its rich supply of essential nutrients, maca contains alkaloids, sterols, tannins, and saponins (Piacente *et al.*, 2002). On the other hand, maca products are claimed to be standardized to 0.6 % macamides and macaenes. This suggests that these compounds are required for the targeted biological activity or that they are used as quality markers.

3. Garlic. Garlic food supplements are used for their antimicrobial, anti-inflammatory, antithrombotic, anticancer, and antiatherosclerotic effects, as well as for their capacity to lower serum lipid levels and ocular pressure. Most of these beneficial effects are referable to a sulfur compound known as allicin although it is often stated that the effects of garlic are due to a synergism of different active ingredients (Schulz, 1998). The intact garlic clove does not contain allicin but rather its precursor, alliin. Alliin is converted to allicin by the enzyme alliinase, when the bulb is cut or crushed (Rubinkov *et al.*, 1994). Allicin is in its turn converted into different sulfur-containing compounds of which more than 100 are known.

4. St John's wort. The major phytochemical constituents of St John's wort are hypericin and pseudohypericin (naaphthodiantrones), and phloroglucinols (hyperforin and adhyperforin). It is often postulated that one or more of these constituents are responsible for the antidepressant effect of St John's wort. Other potentially important constituents include flavonoids (rutin, hyperoside, isoquercitrin, quercitrin and quercetin), chlorogenic acid and amentoflavone (3' 8" biapigenin) (Gray *et al*., 2000); all of which may contribute to the activity of St John's wort (Cui *et al.* 2002).

5. Ginkgo biloba. Ginkgo biloba is mostly used for the improvement of blood circulation. Important constituents of this food supplement are terpene trilactones, namely ginkgolides A, B, C, J and bilobalide, flavonol glucosides, biflavones, proanthocyanidins, alkylphenols,

simple phenolic acids, 4-*O*-methoxypyridoxine and polyprenols (Van beek, 2002). In many commercially available Ginkgo extracts some of these classes of compounds are no longer present.

6. Black radish. Black radish contains glucosinolates and their derivatives (isothiocyanates, nitriles, cyano-epithioalkanes formed during hydrolysis catalysed by myrosinase), essential oils, flavonoids and other polyphenolic compounds. Glucosinolates are of interest because the enzymatically released aglycones are physiologically active compounds (Fenwick *et al.,* 1983). Several authors have proved that these components have strong anticarcinogenic activity via induction of phase II anticarcinogenic enzymes such as glutathione-S-transferases, quinone-reductase and glutathione peroxidase, or modification of the microsomal monooxygenase (cytochrome P450) enzyme system (Kore *et al.,* 1993). In addition, direct antioxidant activity of the glucosinolate degradation products was observed (Wortelboer *et al.,* 1992).

2.2.3.2. Chemical analysis of active ingredients

A literature review of analytical methods for the active ingredients described under section 2.2.3.1 was performed, as a basis for the development of analytical methodologies for the food supplements under investigation. The selected methods are given in Table 7. These methods are currently being optimized for further application to the food supplement matrices. For the black radish based food supplements the analytical method has been fully optimized and applied to the selected samples.

Table 7. Selected analytical methods for the analysis of active ingredients in food supplements

	Analytical method	***Reference***
Blak radish: glucosinolates	HPLC-DAD	ISO 9167-1, 1992
Garlic: allicin	HPLC-UV	Baghalian *et al*., 2005
Ginkgo Biloba: bilobalide, ginkgolide, kaempferol, quercetin	HPLC-UV, HPLC-RI	Van Beek TA *et al*., 2002
Soy isoflavones: daidzein, genistein, glycitein	HPLC-UV	Apers *et al.*, 2004
St John's wort: hyperforin, hypericin	HPLC-UV	Williams *et al.*, 2006
Maca: MTCA, lepidilin A, lepidilin B	CERVA	CERVA

2.2.3.2.1. Analysis of glucosinolates in black radish based food supplements

The determination of the glucosinolates (GL) content in black radish based food supplements was performed using a methodology adapted from the ISO 9167-1-method (ISO 9167-1, 1992). The methodology implied the extraction of GLs (after inactivation of the myrosinase) with a mixture of methanol and water followed by the purification and enzymatic desulfatation on ion-exchange resin. Further, the desulfoderivatives were determined using reversed phase liquid chromatography (RP-LC) with gradient elution and diode array detection (DAD). For the identified GLs, the individual contents were determined by using sinigrin as internal standard and taking into account reported (Wathelet *et al.*, 2004) relative response factors (RRF). A RRF of 1 was arbitrarily applied for the unidentified GLs.

The results obtained (See Table 8) indicated that glucoraphasatin (GRH) is the most significant GL in the food supplements investigated. However, it is present in variable amounts in the different samples. The total GL content was also found to vary from one sample to another. This variation of the total glucosinolate content can be attributed to differences between species and subspecies of the different black radishes from which the food supplements were derived. Different growing conditions could also have contributed to the differences in the glucosinolate content in the different samples (Ciska *et al.*, 2000). For all products investigated, no indication of the GL content was provided by the manufacturer, what made the assessment of product compliance difficult. However, these results clearly demonstrate the need of a control of the quality of the products available on the market. From Table 9, it can be observed that only GRH and glucotropaeolin (GTL) could be identified under the experimental conditions mentioned above. The other compounds showed typical GL UV spectra (Wathelet *et al.*, 2004); however, further confirmation of their identity was not possible due to lack of reference substance. Further coupling of the LC method to mass spectrometry (MS) is planned in order to elucidate the structures of the unidentified peaks. This would also allow to ascertain the peaks already identified through retention time (RT) and UV spectra.

Table 8. Individual and total glucosinolate content : µmol per amount of product corresponding to the manufacturer's daily recommended intake

		Sample N°					
Compound	RRF	43	44	45	53	63	64
UNK1	1	0,11	0,06	0,42	0,24	0,21	0,02
UNK2	1	0,44	0,16	1,06	0,13	0,14	0,11
UNK3	1	0,28	0,11	0,76	0,06	0,10	0,10
UNK4	1	0,20	0,12	1,09	0,04	0,04	0,16
UNK5	1	0,36	0,10	0,54	0,04	0,08	0,09
UNK6	1	0,49	0,12	0,50	0,14	0,12	0,07
UNK7	1	0,25	0,09	0,95	0,01	0,01	0,02
UNK8	1	0,22	0,04	0,12	0,01	0,01	0,02
UNK9	1	0,24	0,06	0,16	0,04	0,06	0,05
DS-GRH	0.4	0,69	0,38	2,27	0,04	0,51	0,26
DS-GTL	0.95	0,27	0,11	0,62	0,10	0,12	0,07
UNK10	1	0,38	0,09	0,29	0,06	0,10	0,03
UNK11	1	0,18	0,11	0,76	0,10	0,12	0,11
Total		**4,11**	**1,50**	**9,55**	**1,00**	**1,61**	**1,09**

Sample N°43 : Arko Radis Noir ; N°44 : Elusan Radis Noir, N°45 : Radis Noir (Fenioux),N°53 : Radis Noir (Diet Natura), N°63 : Radis Noir (Super Diet), N°64 : Radis Noir (Ephyto)

2.2.3.2.2. Analysis of alkaloïds in Maca based food supplements

Alkaloïds analysed. Maca based food supplements were screened for the presence of lepidilin A and lepidilin B. Lepidilins are imidazole alkaloïds with cytotoxic activity, seen as "promising functional ingredients" and as "quality markers" in commercial powders (Jin et al, 2007). AFSSA considers the presence of alkaloïds as potentially dangerous (AFSSA, 2000). Maca based dietary supplements were also screened for the presence of macaridin (3-benzyl-

1,2-dihydro-N-hydroxypyridine-4-carbaldehyde) and (1R,3S)-1-methyltetrahydro-ß-carboline-3-carboxylic acid (MTCA). Macaridin is an alkaloïd and has been shown in Maca tubers (Muhammad et al, 2002), while MTCA, also an alkaloïd, was detected in Maca tubers (Piacente et al, 2002). MTCA occurs in food, wine, beer, etc.. and may be present in human biological tissues and fluids (Herraiz T, 2000).

Methodology. The alkaloïds were extracted with 90 % methanol during 3 hours on an orbital shaker. Every 30 minutes the extract was vortexed. One capsule was extracted with 10 ml 90 % methanol.After centrifugation, the extract was diluted 50 or 100 times (depending on the total amount in one capsule) and injected in a nano-LC-MS (LCQ) . The system is described in Meiring et al, 2002. Briefly, 5 µl of sample was injected (Famos, LC Packings) on a trap column (100 µ ID, 2 cm, 5 µ C-18 Biosphere, flow 5 µl/min, Nanoseparations, The Netherlands) and washed on column during 6min (restrictor closed, valve 6 open). By switching the LCQ valve, the sample was analysed on a nano-column (50 µ ID, 20 cm, 5 µ, C18 Biosphere, Nanoseparations, The Netherlands, flow 0.6 ml/min, restrictor open, valve 6 closed). Solvent A was 5 % acetonitrile, solvent B was 95 % acetonitrile both containing 20 mM formic acid. The gradiënt started at 80/20 and was changed to 20/80 in 10 minutes. It was hold on for 1 minute and then changed to 100 % B. It was hold on for 10 minutes, whereafter the comlumn was equilibrated again at 100 % A during 10 minutes. Detection of the alkaloïds occurred with a LCQ ion trap mass spectrometer. Full MS^2 spectra were recorded. As we have no pure standards, we only compared the extracts among themselves, by comparing the ratio area/amount of supplement extracted.

Results. The results for the determination of lipidilin A and B in maca food supplements can be found in Table 9.

Table 9 : Results of the analysis lepidilin A and B in maca food supplements

Brand	CODA-codification	Normalised area/ µg supplement	
		Lepidilin A	Lepidilin B
Biodynamics bvba	29	10	13
Laboratoires Fenioux sprl	42	5	14
Decola (Vivadis)	46	9	14
Matisson Healthcare	7	100	100
AOV	8	37	24
Dieti natura	52	Not detected	Not detected

Results for lipidilins (Table 9) show that the samples can be divided in three groups :

1. Samples 7 and 8 have the highest amount of lepidilins. Both samples are bought through internet-sites and the products are not notified.
2. Samples 29-42-46 have the lowest amounts of lepidilins. The samples were bought in a drugstore and the products are notified.
3. Sample 52 : no lepidilins were detected. The sample was bought trough the internet and the product is not notified. From the data of this sample, it can be concluded that it does not resemble a maca-extract.

The results for the determination of macaridin and MTCA are presented in Table 10.

Table 10 : Results of the analysis macaridin and MTCA in maca food supplements

Brand	CODA-codification	Normalised area/ µg supplement	
		Macaridin	MTCA
Biodynamics bvba	29	28	Not detected
Laboratoires Fenioux sprl	42	100	Not detected
Decola (Vivadis)	46	16	< 20 ppb
Matisson Healthcare	7	51	Not detected
AOV	8	29	Not detected
Dieti natura	52	51	Not detected

MTCA was detected in 1 sample, at the trace level. There was no relation with any of the other alkaloids analysed. Macaridin was detected in all Maca supplements, with no relation with the lepidilins, as for instance sample 52 contained no lepidilins, but clearly contains macaridin.

2.2.3.3. Calculation of working concentrations

In order to work with plausible intestinal concentrations, we have applied the following dilution hypotheses to the estimated daily intake (EDI) of each selected compound:

- **[Maximal]** (ppb or µg/L): 1L corporal fluid/meal, 1 meal/day
[Maximal] = EDI (µg/person/day)

- **[Minimal]** (ppb or µg/L): 3L corporal fluid/meal, 3 equal meals/day
[Minimal] = [Maximal] / 9

The EDI may be calculated either with values from literature or from packaging. For certain compounds, such as active ingredients from soy isoflavones, the EDI from the diet must be added to the EDI from food supplement consumption. This methodology provided us with an idea of plausible intestinal concentrations. These concentrations will be compared with dose-response curves obtained with *in vitro* models.

A. St John's Wort

Intestinal concentration calculated on the basis of packaging information

Table 9 shows the EDI calculated on the basis of the information on packaging. The content of hypericin is standardized to 0,3% in most food supplements. The content of hyperforin is not specified on the product.

Table 11: EDIs calculated on the basis of the information on packaging

	St John's wort extract (µg)	Hypericin (µg/cap)	recommended dose (caps/day)	Hypericin EDI (µg/person/day)	Hypericin [Maximum] in intestine
Millepertuis (VitaDyne, n°68)		233	3	699	699 ppb → **0,699 ppm**
St Janskruid (Springfield, n°6)	500 000	1 500	2	3 000	3000 ppb → **3 ppm**
Goed Gemoed (Vitamin Health, n°16)	300 000	900	1	900	900 ppb → **0,9 ppm**

0,3%

Intestinal concentration calculated on the basis of information from literature

Table 11 and 12 show EDI calculated on the basis of the quantification of active ingredients in food supplements, according to data from literature. Table 9 shows EDI calculated using data from Li & Fitzloff (2001), whereas table 10 shows EDI calculated using data from Pellati *et al.* (2005).

Table 12.1: EDI calculated on the basis of active ingredient concentrations found in literature (Li & Fitzloff, 2001).

	% in a 650 mg tablet	Compound (mg/tablet)	recommended dose (tabs/day)	EDI (µg/person/day)	Compound [Maximum] in intestine
Hypericin	0,029	0,1885	? ***Hypothesis:3***	565,5	565,5 ppb → **0,5655 ppm**
Hyperforin	0,061	3,965		11 895	11 895 ppb → **11,895 ppm**

Table 12.2 : EDIs calculated on the basis of active ingredient concentrations found in literature (Pellati *et al.*, 2005).

	mg/g	Compound (mg/tablet)	recommended dose (tabs/day)	EDI (µg/person/day)	Compound [Maximum] in intestine
Hypericin	0,62-3,62	0,31-**1,81**	? ***Hypothesis:3***	5 430	5 430 ppb → **5,43 ppm**
Hyperforin	0,7-67,89	0,35-**33,945**		101 835	101 835 ppb → **101,835 ppm**

Hypothesis: 500 mg tablet

From the calculations described above, we can observe that calculated concentrations vary among brands and information from literature. We propose thus a range of plausible intestinal concentrations for hypericin and hyperforin, as follows:

Hypericin (504,46 g/mol)**: 0,5 ppm → 5 ppm (0,991 µM → 9,91 µM)**
Hyperforin (536,79 g/mol)**: 10 ppm → 100 ppm (18,63 µM → 186,3 µM)**

B. Soy isoflavones

Intestinal concentration calculated on the basis of packaging information: food supplement consumers

Table13 shows the EDIs for genistein, daidzein and glycitein calculated on the basis of the available information on packaging. Maximal plausible intestinal concentrations corresponding to a plausible daily intake of each isoflavone diluted in 1L of corporal fluid are presented in Table14.

Intestinal concentration calculated on the basis of literature information

Results (EDIs) of yet published studies helped us to calculate maximal plausible intestinal concentration of soy isoflavones (Table15). However, in most cases the EDI include both genistein and daidzein.

Table13: EDIs of soy isoflavones calculated on the basis of the available information on packaging.

	Soy extract (mg)	% Isof-lavones	Isoflavones (mg)	Genistein (mg/cap)	Daidzein (mg/cap)	Glycitein (mg/cap)	Recommen-ded dose (caps/day)	**Genistein EDI (mg/person/ day)**	**Daidzein EDI (mg/person/ day)**	**Glycitein EDI (mg/person/ day)**
Mega-soja (Springfield, n° 9)	125	40	50	10 (20%)	6 (12%)	-	2	20	12	-
Phyto Soya (Arkopharma, n°18)	-	-	17,5	5,83 *	5,83 *	5,83 *	2	11,66	11,66	11,66
Isoflavone 60 (BeoLife, n° 27)	62,5	40	25	6,25	18,75	-	1	6,25	18,75	-
Bioptimum (Boiron, n°33)	-	-	25	8,33*	8,33*	8,33*	2	16,66	16,66	16,66
Elugyn Fort (Pierre Fabre Santé, n° 34)	250	40	100	33,33 *	33,33 *	33,33 *	1	33,33	33,33	33,33
IsoMex (Metagenics, n° 35)	200	40	80	46 (57,5%)	29 (36,25%)	5 (6,25%)	2	92	58	10
Isoflavones 50 (Orthonat, n°41)	125	40	50	17	35	-	1	17	35	-
Isovon (Orchid Healthcare, n°60)	-	40	60	20 *	20 *	20 *	1	20	20	20
Isoflavones (NetLab. Pharma, n°61)	400	-	35	22,5	7,5	-	2	45	15	-
Meno 24 (NetLab. Pharma, n°62)	400	-	44	29	-	-	1 JOUR	40	-	-
	145	-	16	11	-	-	1 NUIT			
Isoflavones de soja (ephyto, n°65)	-	-	300	100 *	100 *	100 *	6	600	600	600
Soy isoflavones (Natrol, n°66)	-	-	-	16	14	5	4	64	56	20

* Assuming equal percentage of genistein, daidzein and glycitein.

Table14: Maximal intestinal concentration of soy isoflavones after dilution in the bolus.

	Genistein [maximum] in intestine (ppm)	Daidzein [maximum] in intestine (ppm)	Glycitein [maximum] in intestine (ppm)	**Genistein [maximum] in intestine (µM)** *	**Daidzein [maximum] in intestine (µM)** *	**Glycitein [maximum] in intestine (µM)** *
Mega-soja (Springfield, n° 9)	20	12	-	74	47,2	-
Phyto Soya (Arkopharma, n°18)	11,66	11,66	11,66	43,15	45,86	41
Isoflavone 60 (BeoLife, n° 27)	6,25	18,75	-	23,13	73,76	-
Bioptimum (Boiron, n°33)	16,66	16,66	16,66	61,65	65,53	58,6
Elugyn Fort (Pierre Fabre Santé, n° 34)	33,33	33,33	33,33	123,35	131,11	117,23
IsoMex (Metagenics, n° 35)	92	58	10	340,48	228,16	35,17
Isoflavones 50 (Orthonat, n°41)	17	35	-	62,91	137,68	-
Isovon (Orchid Healthcare, n°60)	20	20	20	74	78,67	70,34
Isoflavones (NetLab. Pharma, n°61)	45	15	-	166,54	59	-
Meno 24 (NetLab. Pharma, n°62)	40	-	-	148	-	-
Isoflavones de soja (ephyto, n°65)	600	600	600	2220	2360	2110
Soy isoflavones (Natrol, n°66)	64	56	20	236,8	220,3	70,34

* Genistein 270,2 g/mol; Daidzein 254,2 g/mol; Glycitein 284,3 g/mol.

Table15: EDIs for both soy consumers and non-consumers calculated on the basis of data from published studies.

	EDI (mg/person/ day)	[maximum] in intestine (ppm)	Study	Intake	References
Soy non-consumers	1	1	In 4 European countries (VENUS study)	Genistein + Daidzein	Van Erp-Baart *et al*. (2003)
	1	1	UK	Genistein + Daidzein	Mulligan *et al*. (2007)
	0,03	0,03	France	Genistein + Daidzein: average of men, women and child	AFFSA (2005)
Soy consumers	100	100	Asia	Genistein + Daidzein	Van Erp-Baart *et al*. (2003)
	47	47	Infant fed soy-based formulas	Genistein + Daidzein, glycosides	Setchell *et al.* (1997)
	50	50	Japan	Isoflavones (aglycones)	Messina *et al.* (2006)
	8,6	8,6	Women intake in UK	Genistein + Daidzein,	Mulligan *et al*. (2007)

The following range of maximal intestinal concentrations may be proposed for isoflavone-based food supplement consumers:

Genistein (270,2 g/mol)**: 6 ppm → 92 ppm (23 µM → 340 µM), until 600 ppm**
Daidzein (254,2 g/mol)**: 11 ppm → 58 ppm (45 µM → 228 µM), until 600 ppm**
Glycitein (284,3 g/mol)**: 10 ppm → 33 ppm (35 µM → 117 µM), until 600 ppm**

However, in Asia, high soy consumption strongly contributes to the intake of isoflavones (Table 5). In the particular case of high consumption of both soy and isoflavone-based food supplements, we should be aware that the intake from the diet must be added to the intake from food supplements consumption.

C. Ginkgo biloba

Intestinal concentration calculated on the basis of packaging information

Table 16 shows the EDIs for sesquiterpenes (ginkgolides A, B, C, J, bilobalide) and flavonols (kaempferol, quercetin, isorhamnetin) calculated on the basis of the available information on packaging. Maximal plausible intestinal concentrations corresponding to a plausible daily intake of each compound diluted in 1L of corporal fluid are presented in Table 7.

Intestinal concentration calculated on the basis of literature information

Table 8 show EDI calculated on the basis of the quantification of active ingredients in food supplements, according to data from literature.

Considering results from the two above described methodologies (Table 7 and Table 8), the following range of maximal intestinal concentrations may be proposed for food supplement consumers:

Sesquiterpenes:

Ginkgolide A (408,4 g/mol)**: 0,12 ppm → 7,79 ppm (0,29µM → 19µM)**
Ginkgolide B (424,4 g/mol)**: 0,12 ppm → 10,4 ppm (0,28µM → 24,5µM)**
Ginkgolide C (440,4 g/mol)**: 0,36 ppm → 3,09 ppm (0,83µM → 7µM)**
Ginkgolide J (424,4 g/mol)**: 0,18 ppm → 2,88 ppm (0,44µM → 6,78µM)**
Bilobalide (326,3 g/mol)**: 0,6 ppm → 13,16 ppm (1,9µM → 46µM)**

Flavonols:

Kaempferol (286,24 g/mol)**: 1,33 ppm → 22,4 ppm (4,6µM → 66,3µM)**
Quercetin (338,3 g/mol)**: 1,33 ppm → 81,4 ppm (3,9µM → 250µM)**
Isorhamnetin (316,26 g/mol)**: 1,33 ppm → 19,2 ppm (4,2µM → 60,7µM)**

Table 16: EDIs of terpenes and flavonoids calculated on the basis of the available information on packaging.

	Ginkgo extract (mg)	% Terpenes	% Flavonoids	Terpenes (mg/cap)	Flavonoids (mg/cap)	Recommended dose (caps/day)	**Terpenes EDI (mg/person/day)**	**Flavonoids EDI (mg/person/day)**	**Remark**
Ginkgo (Orthica, n°2)	40	6	24	2,4	9,6	3	7,2	28,8	
Gingko Extra Forte (Bloem, n° 3)	120	6	24	7,2	28,8	2	14,4	57,6	
PC-Ginkgo (Liberty Healthcare, n° 4)	120	-	-	-	-	2	-	-	
Ginkgo 60 (Solgar Vitamins, n° 12)	60	6	24	3,6	14,4	2	7,2	28,8	+ 30 mg non standardized leaf powder extract
Ginkgo Combi (Distributie Care, n° 13)	150 (24% standardized) *	-	-	-	-	1	-	-	
Bio-Biloba (Pharma Nord, n° 14)	100	6	24	6	24	2	12	48	
Ginkgo-Max™ (Good Health, n° 24)	40	-	24	-	9,6	3	-	28,8	
Ginkgo biloba (Parabolic Biologicals, n° 25)	110	-	-	-	12	3	-	36	
Ginkgo 400 (BeoLife, n° 26)	400	-	Min 0,5g/100g	-	2	2	-	4	
Ginkgo (Elusan, Pierre Fabre Santé, n° 30)	200	-	-	-	-	1	-	-	
Ginkgo biloba (FuncioMed, n°31)	60 (24% concentrated) *	-	-	-	-	1	-	-	
Ginkgo biloba (n° 55)	280	-	-	-	-	6	-	-	

* Concentrated or standardized compounds are not specified on the label.

Table17: Maximal intestinal concentration of terpenes and flavonoids after dilution in the bolus.

	Ginkgolides A, B, C, J and bilobalide [maximum] in intestine (ppm) [1]	Kaempferol, quercetin and isorhamentin [maximum] in intestine (ppm) [2]	Ginkgolides A, B, C, J and bilobalide [maximum] in intestine (µM) [1, 3]	Kaempferol, quercetin and isorhamentin [maximum] in intestine (µM) [2, 3]
Ginkgo (Orthica, n°2)	1,44	9,6	Ginkgolide A: 3,52 Ginkgolide B: 3,39 Ginkgolide C: 3,26 Ginkgolide J: 3,39 Bilobalide: 4,41	Kaempferol: 33,53 Quercetin: 28,37 Isorhamnetin: 30,35
Gingko Extra Forte (Bloem, n° 3)	2,88	19,2	Ginkgolide A: 7,05 Ginkgolide B: 6,78 Ginkgolide C: 6,53 Ginkgolide J: 6,78 Bilobalide: 8,82	Kaempferol: 67,07 Quercetin: 56,75 Isorhamnetin: 60,7
PC-Ginkgo (Liberty Healthcare, n° 4)	-	-	-	-
Ginkgo 60 (Solgar Vitamins, n° 12)	1,44	9,6	Ginkgolide A: 3,52 Ginkgolide B: 3,39 Ginkgolide C: 3,26 Ginkgolide J: 3,39 Bilobalide: 4,41	Kaempferol: 33,53 Quercetin: 28,37 Isorhamnetin: 30,35
Ginkgo Combi (Distributie Care, n° 13)	-	-	-	-
Bio-Biloba (Pharma Nord, n° 14)	2,4	16	Ginkgolide A: 5,87 Ginkgolide B: 5,65 Ginkgolide C: 5,44 Ginkgolide J: 5,65 Bilobalide: 7,35	Kaempferol: 55,89 Quercetin: 47,29 Isorhamnetin: 50,59
Ginkgo-Max™ (Good Health, n° 24)	-	9,6	-	Kaempferol: 33,53 Quercetin: 28,37 Isorhamnetin: 30,35
Ginkgo biloba (Parabolic Biologicals, n° 25)	-	12	-	Kaempferol: 41,92 Quercetin: 35,47 Isorhamnetin: 37,94
Ginkgo 400 (BeoLife, n° 26)	-	1,33	-	Kaempferol: 4,64 Quercetin: 3,93 Isorhamnetin: 4,2
Ginkgo (Elusan, Pierre Fabre Santé, n° 30)	-	-	-	-
Ginkgo biloba (FuncioMed, n°31)	-	-	-	-
Ginkgo biloba (n° 55)	-	-	-	-

[1] Assuming equal amount of ginkgolide A, B, C, J and bilobalide in global terpene EDI (EDI/5 is diluted in 1L of corporal fluid).

[2] Assuming equal amount of kaempferol, quercetin and isorhamnetin in global flavonoid EDI (EDI/3 is diluted in 1L of corporal fluid).

[3] Ginkgolide A 408,4 g/mol; Ginkgolide B 424,4 g/mol; Ginkolide C 440,4 g/mol; Ginkgolide J 424,4 g/mol; bilobalide 326,3 g/mol; Kaempferol 286,24 g/mol; quercetin 338,3 g/mol; isorhamnetin 316,26 g/mol.

Table 18: EDI calculated on the basis of active ingredient concentrations found in literature.

	Compound (µg/cap)		Recommen-ded dose (caps/day) [1]	**EDI (µg/person/day)**		**Compound [maximum] in intestine (ppm)**		**Compound [maximum] in intestine (µM) [2]**	
	Min.	Max.		Min.	Max.	Min.	Max.	Min.	Max.
Dubber and Kanfer (2006)									
Ginkgolide A	304,3	3445	2	608,6	6890	0,6086	6,89	1,49	16,87
Ginkgolide B	176	1910	2	352	3820	0,352	3,82	0,83	9,00
Ginkgolide C	183	1547,5	2	366	3095	0,366	3,095	0,83	7,03
Ginkgolide J	93,7	641,5	2	187,4	1283	0,1874	1,283	0,44	3,02
Bilobalide	314,7	1358,25	2	629,4	2716,5	0,6294	2,7165	1,93	8,33
de Jager *et al.* (2006)									
Ginkgolide A	774	7790	1	774	7790	0,774	7,79	1,90	19,07
Ginkgolide B	648	4680	1	648	4680	0,648	4,68	1,53	11,03
Ginkgolide C	666	1800	1	666	1800	0,666	1,8	1,51	4,09
Ginkgolide J	540	980	1	540	980	0,54	0,98	1,27	2,31
Bilobalide	1118,6	3330	1	1118,6	3330	1,1186	3,33	3,43	10,21
Mesbah *et al.* (2005)									
Ginkgolide A	60	2420	2	120	4840	0,12	4,84	0,29	11,85
Ginkgolide B	60	5200	2	120	10400	0,12	10,4	0,28	24,51
Bilobalide	840	6580	2	1680	13160	1,68	13,16	5,87	45,98
Kaempferol	2000	11220	2	4000	22440	4	22,44	11,83	66,34
Quercetin	2660	40730	2	5320	81460	5,32	81,46	16,30	249,65

[1] Recommended dose unknown in Dubber and Kanfer (2006) and Mesbah *et* al. (2005). A quantity of 2 recommended caps/day has been used for calculation.

[2] Ginkgolide A 408,4 g/mol; Ginkgolide B 424,4 g/mol; Ginkolide C 440,4 g/mol; Ginkgolide J 424,4 g/mol; bilobalide 326,3 g/mol; bilobalide 326,3 g/mol; Kaempferol 286,24 g/mol; quercetin 338,3 g/mol; isorhamnetin 316,26 g/mol.

2.3. Biological *in vitro* assays

In a first phase of the project active ingredients of the selected food supplements were tested with the different *in vitro* assays. The selection of the active ingredients is based on literature and availability from commercial suppliers.

2.3.1. Assessment of stress gene responses.

Toxicity and basic mode of action (MOA) characterisation of the chosen active ingredients are the principal tasks performed at the University of Antwerp in phase I of the project. A battery of genetically modified *Escherichia coli* (Orser et al., 1995) and human hepatoma cells (HepG2) (Todd et al., 1995) with stress gene promoters were used to gain insight in the stress-related mode of action of the active ingredients of the selected food supplements. Previous to the MOA characterisation general toxicity was evaluated for *Escherichia coli* (growth inhibition) and human hepatoma cell lines, HepG2 (cytotoxicity). Based on the toxicity data appropriate concentration ranges were determined for the bacterial gene expression assay.

General toxicity

For growth inhibition/cytotoxicity curves, best fit regression curves were obtained with SigmaPlot 8.0 (SPSS Inc.). According to the selected regression model, XC_{50} values were calculated. Based on the bacterial growth inhibition experiments IC_{50} (concentration at which 50% of the growth was inhibited) concentrations were calculated. The same compounds were tested with the HepG2 cells resulting in clear dose response curves with calculations of LC_{50} (concentration at which 50 % of the cells are dead) concentrations.

Table 19: Growth inhibition and cytotoxicity results represented as IC_{50} (bacteria) and LC_{50} LC_{20} and LOEC (HepG2) values. Values are calculated based on curve fitting, if no 100 % inhibition or mortality was reached the highest tested concentration is noted. All concentrations are expressed in µM exepted for garlic oil (µg/ml).

Compound	IC_{50}	LC_{50}	LC_{20}	LOEC
DL-sulforaphane	103	16.9	11.61	6.25
L-sulforaphane	183	34.6	16.34	12.5
Garlic oil	4.42 µg/ml			
Hypericin	76.33	31.56	22.70	24.78
Hyperforin	> 18.6	2.44	2.34	2.34
Daidzein	>393	> 393	-	> 393
Genistein	81.03	> 370	100.39	92.51

As can be seen from Table 19, differences in toxicity are found for the different active ingredients present in one food supplement, for instance for two of the active ingredients of Saint-Johns Wort the LC_{50} value for hypericin is ten times higher than for hyperforin. The estimated daily intake (EDI) for hyperforin (11 – 101 ppm) is far above the calculated LC_{50} concentration of 1.31 µg/ml , on the contrary the EDI for hyperficin (0.5 – 5 ppm) is below the LC_{50} of the corresponding compounds (15.9 µg/ml). It has been stated previously that hyperforin is a promising new anticancer agent since it inhibits growth of different tumor cell lines, like the used HepG2 cells (Schempp et al., 2002, Beerhues, 2006). The results at the level of toxicity for the HepG2 cells and the bacteria are not directly comparable, given that bacteria are less

sensitive to most compounds that are not anti-microbial. Relative high IC_{50} values are found after exposure to hypericin and hyperforin and low LC_{250} values are found after exposure of the HepG2 cells, this can be explained by the findings that hypericin and hyperforin do not exhibit antibacterial effects against gram negative bacteria like *E. coli*.

Daidzein and genistein both soy isoflavones showed low toxicity, indicated by the absence of growth inhibition/mortality. Therefore no XC_{50} values could be determined.

The calculated IC_{50} value for garlic oil of 4.42 µg/ml is remarkably low. Antimicrobial properties of garlic oil have already been reported, and the observed IC_{50} may confirm this observation. Its concentration range is comparable to calculated IC_{50} values for some frequently used antibiotics like ampicillin (IC_{50} 3 µg/ml) or tetracycline (IC_{50} (5 µg/ml). Interesting further challenges might be the unravelling of mechanism of growth inhibition, the observed gene expresson profile might be indicative for this

Mode of action

Target genes are indicated in table 20.

Based on the growth inhibition experiments, the appropriate concentration ranges were selected. 20 % growth inhibition was selected as the highest concentration in a ½ serial dilution with 7 concentrations and each experiment was conducted in triplicate.

Table 21: Table with the different endpoint classes, with the corresponding stress genes that can be induced with the bacterial gene expression assay. The significantly induced genes are given; between brackets is the height of the respective fold induction

Compound	Singificant induced genes			
	oxidative damage KatG, Zwf, Soi28, Nfo, MerR	**Membrane damage** MicF, OsmY	**cellular stress** UspA, ClpB	**DNA damage** RecA, UmuDC, Ada, DinD, SfiA
DL-sulforaphane (1 – 60 µM)	KatG (1.28), MerR (2.19)	OsmY (2.85)	ClpB (1.31)	DinD (2.21)
L-sulforaphane (0.195 – 12.5 µM)	KatG (1.24), MerR (72.1)	OsmY (2.14)	0	DinD (1.71)
Garlic oil (0.005 – 0.3 µg/ml	KatG (1.24)	OsmY (1.28)	ClpB (1.13)	SfiA (1.19)
Hypericin (0.391 - 25 µg/ml)	0	0	0	0
Hyperforin (0.156 - 10 µg/ml)	0	0	0	0
Daidzein (0.781 – 50 µg/ml)	0	0	0	0
Genistein (0.195 – 12.5 µg/ml)	MerR (1.81), Soi28 (1.70) Nfo (1.36)	0	0	DinD (1.74)

In Table 21, a general overview of the obtained bacterial stress gene profiles is given, the general mode of action of the active compounds is characterised by the height of induction and the number of induced stress genes. A number of the selected active ingredients did not show any significant inductions, this is the case for hypericin, hyperforin and daidzein. The known mode of action of St-Johns Wort - one of the most frequently studies medicinal plants - is mainly based on hyperforin. Its major pharmacological activities are situated at the level of unique eukaryotic

systems like neurotransmittor reuptake inhibition, Cyp1A1 inhibition, anti-tumoral properties, all specific MOA which cannot be evaluated through a bacterial system. The eukaryotic assay that will be performed in the second part will provide more insight into these specific MOA.

The enantiomers of sulforaphane that were tested show clear and remarkable differences in especially the height of induction of MerR, 2.19 for DL-sulforaphane and 72.1 for L-sulforaphane. The high induction of MerR is in accordance with previous findings with human hepatoma cells (HepG2); after exposure to sulforaphane they observed elevated metallothionein (MT) expression, intracellular proteins that bind heavy metals with high affinity (Yeh et al., 2005). The functions of MTs are uncertain, but they can detoxify heavy metals, provide a reserve of zinc and protect against oxidative stress. The accordance between MTs and the prokaryotic MerR promoter expression has been found previously for heavy metals (Cadmium, Zinc and Mercury), however we have been the first to report the accordance between oxidative DNA damage at the level of MerR/MTs.

2.3.2. Assessment of hormonal and dioxin-like activities

2.3.2.1. Principle of tools:

Reporter gene assays were used to assess a broad spectrum of biological activities of food supplement's active ingredients, such as dioxin-like, anti-dioxin, steroid hormonal-like and anti-steroid hormonal activities. These biological assays are based on the use of reporter cells harboring an endogenous/exogenous "ligand-dependent transcription factor" and a stable genetic construct consisting of a specific sequence of DNA, a promoter and the luciferase reporter gene. We used six reporter cell lines. Two are dioxin-sensitive cells and four are steroid hormonal-sensitive cells. The first group of cells owns the Aryl hydrocarbon receptor (AhR) and the second group owns different specific steroid hormone receptors. The principle of reporter gene assays is shown in the *figure 1*.

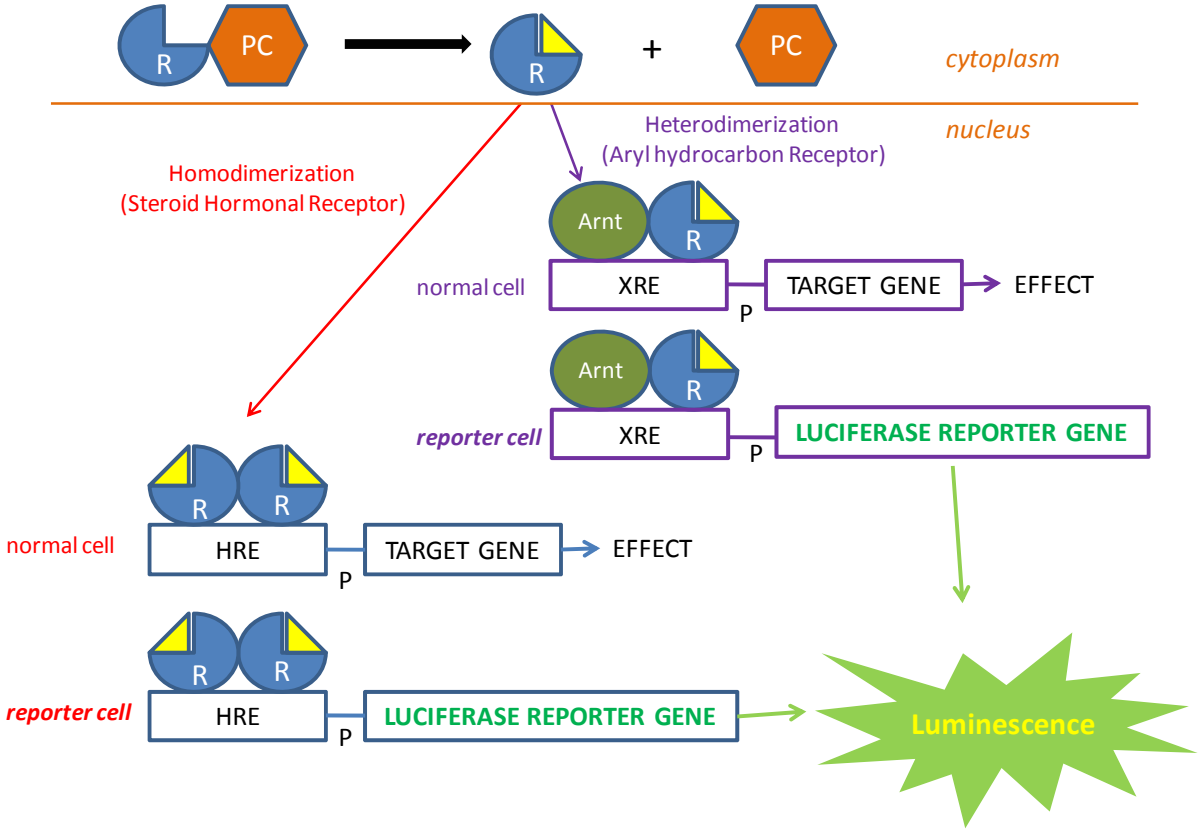


Figure 1: principle of reporter gene assays taking advantages of the AhR and steroid receptors pathways. R= receptor, PC= protein complex, XRE= Xenobiotic Responsive Element, HRE= Hormone responsive Element, P= promoter, Arnt= Aryl receptor nuclear translocator. Yellow triangle is the ligand.

The AhR and the steroid hormonal receptors pathways are very similar. Briefly, the inactive receptor is located in the cytoplasm with a protein complex. Upon binding of ligand, the receptor crosses the nucleus membrane and homodimerizes, in the case of steroid hormone receptor, and heterodimerizes with the Arnt protein, in the case of AhR. This dimer binds to a specific sequence of DNA (XRE or HRE, see figure 1). In normal cells, target genes are transcripted and different effects occur in the cells. In reporter cells, the product of the luciferase reporter gene produce a luminescence, after addition of substrates, which is directly correlated with the ligand concentration (Scippo *et al.*, 2004; Willemsen *et al.*, 2002, 2004 and 2005).

2.3.2.2. Experimental

For each reporter cell line, a reference curve was run in parallel of the tested active ingredients. Cells were exposed with one active ingredient (**agonistic assays**) or with a mixture of the reference substance and each active ingredient (**antagonistic assays**). Results are expressed in relative response. For that, RLUs (Relative Light Units of luminescence), from active ingredient exposition were compared to RLUs measured with a concentration of inducer (reference ligand) producing a maximal luminescence in the cells. An overview of the characteristics of each cellular assay is shown in the *table 23* (genetic construct, the exposure time, the reference substance and the concentration of inducer to assess the antagonistic activity).

Table 23: characteristics of each "cellular assay"

	Name	Species/tissue origin	Genetic construct			Receptor	exposure time	reference substance	Concentration of inducer for antagonistic assays
			specific sequences of DNA	promoter	reporter gene				
Dioxin-sensitive cells	**H4IIE-XRE**	rat hepatoma cells	4 XRE	HSV-TK	luciferase	AhR (endogenous)	6h and 24h	TCDD	300 pM
	HepG2-XRE	human hepatoma cells	4 XRE	HSV-TK	luciferase	AhR (endogenous)	6h and 24h	TCDD	10 nM
Steroid hormonal-sensitive cells	**MCF7-ERE**	human breast tumour cells (from MCF-7 cell line)	1 ERE	vitellogenin	luciferase	ER (endogenous)	24h	17 β-estradiol	5 nM
	TM-Luc	human breast tumour cells (from T-47D cell line)	4 HRE	MMTV	luciferase	PR (endogenous)	48h	progesterone	3.2 µM
	TARM-Luc	human breast tumour cells (from T-47D cell line)	4 HRE	MMTV	luciferase	AR (exogenous)	48h	boldenone	7 nM
	TGRM-Luc	human breast tumour cells (from T-47D cell line)	4 HRE	MMTV	luciferase	GR (exogenous)	48h	dexamethasone	0.1 µM

2.3.2.3. Results from exposition of "soy isoflavones active ingredients" to dioxin-sensitive cells

Agonistic assays

All experiments were carried out with a range of concentrations from 2.5 µM to 80 µM. Each isoflavone was exposed to the cells during 6h and 24h to assess the dioxin-like activity (agonistic activity). H4IIE-XRE is from rat hepatoma cells and then, harbors the rat Ah receptor (rAhR). HepG2-XRE is from human hepatoma cells and then, expresses the human Ah receptor (hAhR). The results of two independent experiments are shown in the *table 24*.

Table 24: Agonistic activities of isoflavones in dioxin-sensitive cells

	rAhR 6h	rAhR 24h	hAhR 6h	hAhR 24h
Daidzein	**++ (20 µM)**	**+ (40 µM)**	-	-
Glycetein	**++ (35 µM)**	-	-	-
Genistein	**++ (40 µM)**	-	-	-

Legend: -: no activity; +: weak activity (<25% of the TCDD maximal response); ++: medium activity (75% >effect >25%).

Isoflavones exerted "medium" agonistic activities in rat hepatoma cells when exposed to the cells during 6h but no more after 24h (except a "subsisting" weak activity for daidzein). In opposite, no activity was measured in human hepatoma cells. These results suggest several things: The isoflavones are able to induce the AhR pathway in rat but in a transient way. The discrepancy between rat and human cells could result from the difference of cell sensitivity towards dioxins. We determined the concentration of half-maximal response (EC50) of TCDD for both rat and human hepatoma cell lines. These values are 48 pM and 800 pM (24h) for H4IIE-XRE and HepG2-XRE, respectively, showing a lower sensitivity of HepG2-XRE cells towards TCDD. In addition, the difference could result in the species origin of the Ah receptor.

Antagonistic assays

Antagonistic assays were carried out to measure an inhibition of the AhR pathway with isoflavones when cells were previously induced by the reference ligand, such as TCDD. High concentrations of TCDD were applied to the cells to induce a maximal activity (100%): 300 pM for H4IIE-XRE and 10 nM for HepG2-XRE. In the same time, increasing concentrations

of isoflavones (from 2.5 to 80 µM) were added to the cells. Isoflavones were exposed to the cells during 6h and 24h to assess the influence of the time exposure.

Table 25: antagonistic activities of isoflavones in dioxin-sensitive cells

	rAhR 6h	rAhR 24h	hAhR 6h	hAhR 24h
Daidzein	**>100%**	**>100%**	-	-
Glycetein	-	-	-	-
Genistein	**>100%**	-	**+ (40 µM)**	**+ (20 µM)**

Legend: -: no activity; +: antagonistic activity (< 75% of the TCDD maximal response); >100%: additive or synergic activity.

Results of "antagonistic assays" showed that some mixtures "isoflavone + TCDD" exerted an additive or synergic activity in rat cells (table 24). To determine if the response was synergic or additive, the results of the mixtures "TCDD + isoflavone" had to be comparing with the results from exposition of isoflavone alone, as shown in *figure 2*.

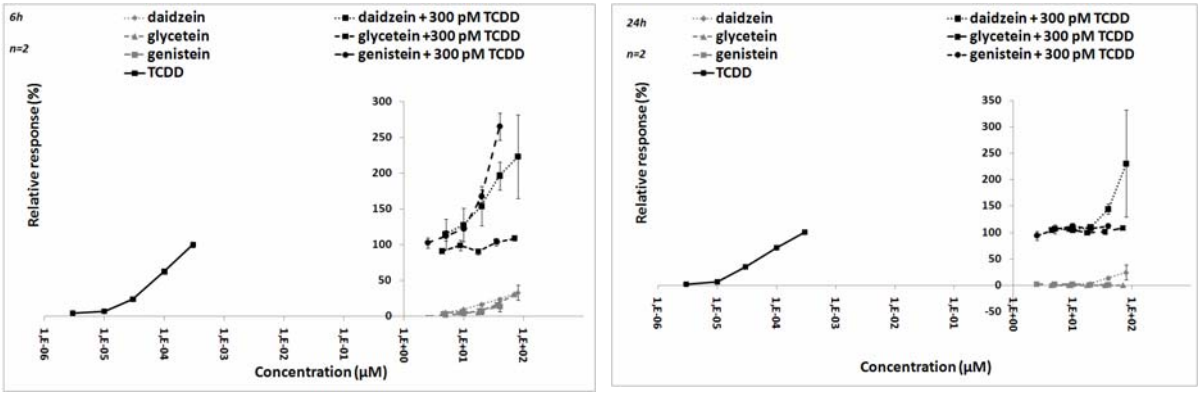


Figure 2: Comparison of mixtures "isoflavone + TCDD" and agonistic activities of isoflavones in rat hepatoma cells H4IIE-XRE. On the left: exposure time of 6h. On the right: exposure time of 24h.

The figure 2 shows a synergy between "daidzein and TCDD" (6h) because the sum of relative responses (calculated from exposition of TCDD alone (100%) and from daidzein exposition (20% at 40 µM)) is lower than the relative response measured from exposition of the mixture "daidzein (40 µM) + TCDD" (200%). A synergy was also observed between "genistein and TCDD" (6h). In human hepatoma cells HepG2-XRE, only genistein (from 40 µM to 80 µM, exposure time of 6 and 24h) was able to decrease the maximal response induced by 10 nM of TCDD (antagonistic activity of genistein) (*figure 3*).

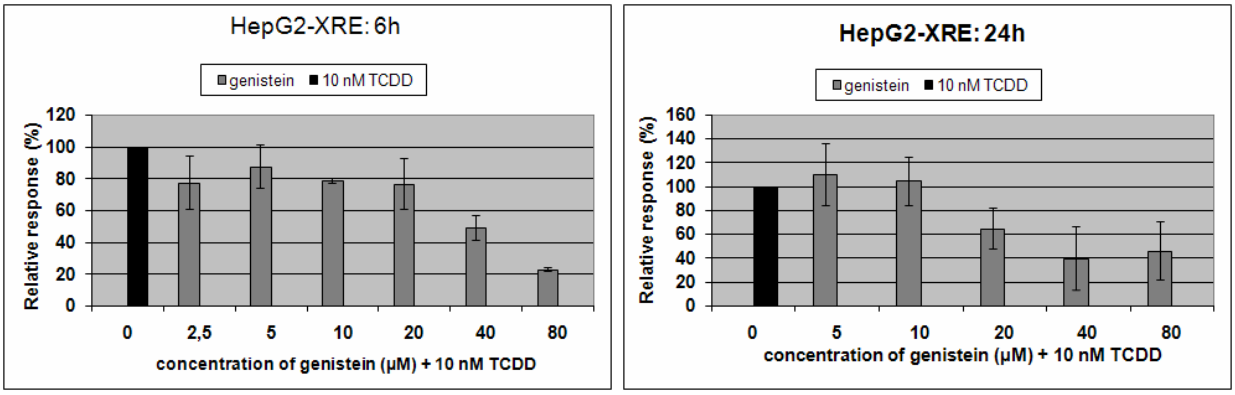


Figure 3: antagonistic activity of genistein in human hepatoma cells HepG2-XRE. On the left: exposure time of 6h. On the right: 24h of exposure time.

2.3.2.4. Results from exposition of "soy-isoflavones active ingredients" to hormonal-sensitive cells

Agonistic assays

All experiments were carried out with a range of concentrations from 2.5 µM to 80 µM. Each isoflavone was exposed, during 24h, to estrogen-sensitive cells (MCF7-ERE) and, during 48h, to the others hormonal-sensitive cells (TM-Luc, TARM-Luc, TGRM-Luc) in order to assess the hormonal-like activities (agonistic activities). All the cells are from human breast tumour cell line. The results of two independent experiments were compared to the maximal response obtained with the reference ligand (dehydrotestosterone: hAR, progesterone: hPR, estradiol: hER and dexamethasome: hGR) and are shown in the *table 26*.

Table 26: Agonistic activities of isoflavones in hormonal-sensitive cells

	hAR 48h	hPR 48h	hGR 48h	hER 24h
Daidzein	-	-	-	**+++ (2 µM)**
Glycetein	-	-	-	**+++ (18 µM)**
Genistein	-	-	-	**+++ (1.2 µM)**

Legend: -: no activity; +: weak activity (<25% of the inducer maximal response); ++: medium activity (75% >effect >25%); +++: strong activity (> 75%).

We concluded that isoflavones have estradiol-like activities (*figure 4*) and then could be classified as phytoestrogens (already described in literature).

Antagonistic assays

Antagonistic assays were carried out to measure an inhibition of the steroid hormonal pathways with isoflavones when cells were previously induced by the reference ligand. High concentrations of inducer (see "table 1") were applied to the cells to induce a maximal activity (100%). In the same time, increasing concentrations of isoflavones (from 2.5 to 80 µM) were added to the cells. The results of two independent experiments were compared to the maximal response obtained with the reference ligand (dehydrotestosterone: hAR, progesterone: hPR, estradiol: hER and dexamethasome: hGR) and are shown in the *table 27.*

Table 27: antagonistic activities of isoflavones in hormonal-sensitive cells

	hAR 48h	hPR 48h	hGR 48h	hER 24h
Daidzein	**> 100%**	**+ (40 µM)**	**+ (80 µM)**	**>100%**
Glycetein	-	**+ (40 µM)**	**+ (70 µM)**	**>100%**
Genistein	-	**+ (20 µM)**	**+ (40 µM)**	**>100%**

Legend: -: no activity; +: antagonistic activity (< 75% of the TCDD maximal response); >100%: additive or synergic activity.

Results showed that mixture "daidzein + dehydrotestosterone" and each mixture of "isoflavone + estradiol" produced a relative response higher than 100%. We concluded a synergy between daidzein and DHT, because when daidzein was applied, alone, on the cells, it didn't produce any response and when mixture of "daidzein and DHT" was applied on the cells, we measured a response of 160%. For each isoflavone, an inhibition of the progestagen and the glucocorticoid pathways occurred. In estradiol-sensitive cells MCF7-ERE, the response was already more than 100 % when each isoflavone was exposed to the cells (see table 4). The "agonistic" and the mixtures "estradiol + isoflavone" dose-response curves obtained in estrogen-sensitive cells are compared in the *figure 4*.

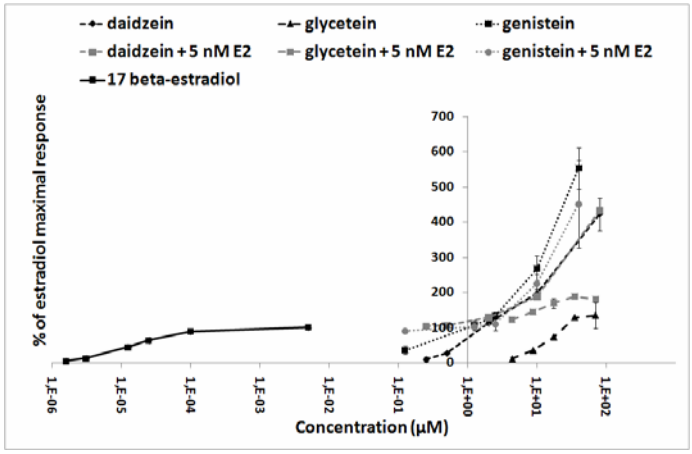


Figure 4: Comparison of dose-response curves obtained from isoflavone exposition and mixture "isoflavone + estradiol" exposition (24h) to estrogen-sensitive cells MCF7-ERE.

2.3.2.5. Results from exposition of "ginkgo biloba active ingredients (GBAI)" to dioxin-sensitive cells

Agonistic assays

All experiments were carried out with a range of concentrations from 1.5 µM to 60 µM. Each GBAI was exposed to the cells during 6h and 24h to assess the dioxin-like activity (agonistic activity). The results of two independent experiments are shown in the *table 28*.

Table 28: agonistic activities of GBAI in dioxin-sensitive cells

	rAhR 6h	rAhR 24h	hAhR 6h	hAhR 24h
Ginkgolide A	-	-	-	-
Ginkgolide B	-	-	-	-
Ginkgolide C	-	-	-	-
Ginkgolide J	**++ (24 µM)**	-	-	-
Bilobalide	-	-	**+ (30 µM)**	-
Kaempferol	-	-	-	-
Quercetin	-	-	**+ (20 µM)**	-
Isorhamnetin	-	-	-	-

Legend: -: no activity; +: weak activity (<25% of the inducer maximal response); ++: medium activity (75% >effect >25%); +++: strong activity (> 75%).

As shown in table 6, only ginkgolide J exerted a medium agonistic activity (from 24 µM) in rat hepatoma cells H4IIE-XRE, when exposed to the cells during 6h. Bilobalide and quercetin had weak agonistic activity when exposed to the human hepatoma cells HepG2-XRE during 6h (from 30 µM for bilobalide and from 20 µM for quercetin). These results suggest that the dioxin-like activities of some GBAI are rather weak and transient in these cells.

Antagonistic assays

All experiments were carried out with a range of concentrations from 1.5 µM to 60 µM of GBAI as explained for isoflavones. Results from two independent experiments are shown in the Table 29.

Table 29: antagonistic activities of GBAI in dioxin-sensitive cells

	rAhR 6h	rAhR 24h	hAhR 6h	hAhR 24h
Ginkgolide A	-	-	-	-
Ginkgolide B	-	-	-	-
Ginkgolide C	-	-	-	-
Ginkgolide J	-	-	-	-
Bilobalide	-	-	**+ (60 µM)**	-
Kaempferol	-	-	**+ (4 µM)**	**+ (18 µM)**
Quercetin	**+ (40 µM)**	**+ (40 µM)**	**+ (5 µM)**	**+ (40 µM)**
Isorhamnetin	**+ (32 µM)**	-	**+ (4 µM)**	**+ (20 µM)**

Legend: -: no activity; +: antagonistic activity (< 75% of the TCDD maximal response)

Some of the flavonoids group (kaempferol, quercetin and isorhamnetin) displayed antagonistic activities on AhR pathway (more important when exposed to the cells during 6h than 24h). In addition, bilobalide displayed also antagonistic activity in human hepatoma cells but from 60 µM.

2.3.2.6. Results from exposition of "ginkgo biloba active ingredients (GBAI)" to hormonal-sensitive cells

Agonistic assays

All experiments were carried out with a range of concentrations from 1.5 µM to 60 µM. Each GBAI was exposed to the cells as explained for isoflavones. The results of two independent experiments are shown in the *table 30*.

Table 30: agonistic activities of GBAI in hormonal-sensitive cells

	hAR 48h	hPR 48h	hGR 48h	hER 24h
Ginkgolide A	/	-	-	-
Ginkgolide B	/	-	-	-
Ginkgolide C	/	-	-	-
Ginkgolide J	/	-	-	-
Bilobalide	-	-	-	-
Kaempferol	/	-	/	**+++ (9 µM)**
Quercetin	/	-	/	**++ (40 µM)**
Isorhamnetin	-	-	-	**+++ (16 µM)**

Legend: -: no activity; +: weak activity (< 25% of the inducer maximal response); ++: medium activity (75% > effect >25%); +++: strong activity (> 75%); /: not yet tested.

Results showed that only flavonoids exerted estrogen-like activities.

Antagonistic assays

All experiments were carried out with a range of concentrations from 1.5 µM to 60 µM. Each GBAI was exposed to the cells as explained for isoflavones. The results of two independent experiments are shown in the *table 30*.

Table 31: antagonistic activities of GBAI in hormonal-sensitive cells

	hAR 48h	hPR 48h	hGR 48h	hER 24h
Ginkgolide A	/	-	-	-
Ginkgolide B	/	-	-	-
Ginkgolide C	/	-	-	-
Ginkgolide J	/	-	-	-
Bilobalide	-	-	-	-
Kaempferol	/	**+ (9 µM)**	/	**> 100%**
Quercetin	/	**+ (20 µM)**	/	**-**
Isorhamnetin	**+***	**+ (8 µM)**	**+***	**-**

Legend: -: no activity; +: antagonistic activity (< 75% of the TCDD maximal response); /: not yet tested. +*: visual cytotoxicity under the microscope.

As shown in table 31, the group of flavonoids (kaempferol, quercetin and isorhamnetin) exerted antagonistic activities in progestagen-sensitive cells TM-Luc. In androgen and glucocorticoid-sensitive cells, isorhamnetin seems to display antagonistic activities but we observed cytotoxicity under the microscope. As measured in agonistic assay, kaempferol exerted higher relative response than 100% when it is exposed to the cells with estradiol.

2.3.2.7. Conclusions

Almost of the "soy isoflavone's and ginkgo biloba's active ingredients" having activities of AhR pathway and steroid hormonal pathways modulation own to the group of **flavonoids** (daidzein, glycetein, genistein, quercetin, isorhamnetin and kaempferol. We already published some data about modulation of AhR pathway by flavonoids (Van der Heiden *et al*., 2008).

2.3.3. Assessment of the impact of FS active ingredients on a human intestinal cell system

Methodology

Biological model. In this project, we used the human colon adenocarcinoma cell line Caco-2 (ATCC, Rockville, MD) (figure 5), a well-known *in vitro* model of the intestinal barrier, in order to study toxicological effects of selective active ingredients contained in food supplements. In the first phase of the project, we worked with standards, before exposing cells to food supplement extracts and mixtures.

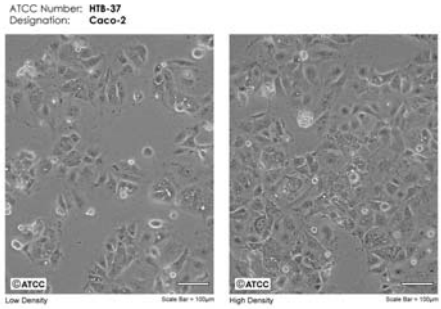


Figure 5: Proliferating Caco-2 cells (ATCC, Rockville, MD)

Caco-2 cells were used between passage 26 and 46 and routinely grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% foetal bovine serum (FBS), 0.1 mM non-essential amino acids and 4mM L-glutamine. Cells were incubated at 37°C in a humidified chamber with 5% (v/v) CO_2, with media change every 2 days.

Chemicals. Quercetin dihydrate, kaempferol, hyperforin, daidzein, glycitein, (-)-bilobalide, ginkgolides A, B, C and J and benzo(a)pyrene (B[a]P) were purchased from Sigma-Aldrich (S^t Louis, MO). Hypericin, isorhamnetin and genistein were from Extrasynthèse (Genay, France).

Impact on cell proliferation (MTS assay). The colorimetric 3-(4,5 dimethylthiazol-2-*yl*)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed to assess the effect of active ingredients on Caco-2 cells proliferation. Cells, seeded in 96-well plates, at a density of 10,000 cells/cm^2, were incubated, 24 h after seeding, with the active ingredient at different concentrations for 48 h. The MTS assay was carried out as described by the manufacturer's instructions (Promega, Madison, WI) with formazan production detected at 500 nm (Spectracount, Packard, Warrenville, IL).

Cytotoxicity (LDH assay). This test, purchased as a kit (Cytotoxicity Detection Kit, Roche diagnostics, Mannheim, DE), measures in the extracellular media the activity of lactate dehydrogenase (LDH), an enzyme released from the cytosol of damaged or necrosed cells. Caco-2 cells were inoculated at 40,000 cells/cm^2 and cultivated until 16-days post-confluence in 48-well plates with culture medium changes 3 times per week. Cells were then incubated with the active ingredients at a determined concentration for 24 h or 6h and the supernatant was collected. Maximal LDH release was obtained by exposing the cells to 1 % (v/v) Triton X-100. The reduced formazan reaction product was measured at 490 nm, with a 690 nm reference wavelength.

Impact on CYP1A-dependent 7-ethoxyresorufin dealkylase (EROD) activity. This assay is based on the *O*-dealkylation of 7-ethoxyresorufin to resorufin, involving the 1A isoform of cytochrome P-450. Caco-2 cells were inoculated at 40,000 cells/cm^2 and cultivated until 16-days post-confluence in 48-well plates with culture medium changes 3 times per week. Cells were then incubated with the active ingredients at a determined concentration for 24 h and 6h before the EROD assay in the presence or absence of benzo[a]pyrene (B[a]P), a well-known CYP1A1 inducer. Medium was collected for cytotoxicity assay (LDH leakage).

Cells were rinsed with PBS and incubated for 1 h at 37°C with 5 µM 7-ethoxyresorufin (EROD) (Sigma-Aldrich, S^t Louis, MO) in phenol red-free DMEM. Fluorescence was measured, in the supernatants, using a fluorometer (Fluoroskan Ascent, Thermo Electron Corporation, Vantaa, Fid) with excitation and emission wavelengths of 530 and 585 nm, respectively. The cell protein content was determined with the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich).

Impact on CYP3A4-dependent activity. This assay is based on the conversion of testosterone to 6β-(OH)-testosterone by the CYP3A4 isoform and detection of metabolites by HPLC. Caco-2 cells were inoculated at 40,000 cells/cm^2 and cultivated in 12-well plates in DMEM supplemented with 5% FBS, with culture medium changes 3 times per week.

- *Inhibitory effect on CYP3A4 activity*. In Caco-2 cells, previous CYP3A4 activity induction by 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3) is needed to study inhibitory effects by pure compounds. From day 8 to 21, 0,5 µM 1α,25-$(OH)_2$-D3 (Fluka, Sigma-Aldrich) was added to the culture medium. At day 21, cells were rinsed with PBS and incubated with 500 µM testosterone (Sigma-Aldrich) diluted in HBSS (*Hank's Balanced Salt Solution*) pH 7,4 and with the active ingredients at determined concentration for 3h, at 37°C.
- *Inducing effect on CYP3A4 activity*. From day 8 to 21, cells were incubated with pure compounds at determined concentration, with medium change 3 times per week. At day 21, cells were incubated during 3h with 500µM testosterone diluted in HBSS pH 7,4.

Supernatants were subjected to HPLC analysis for 6β-(OH)-testosterone detection. After 0.45 µm filtration, 20 µl of cell medium were injected into the HPLC-system (Thermo Separation Products, San Jose, CA) consisting of a P 4000 pump, equipped with an AS 3000 autosampler injector, a SN 4000 interface, a spectra-physics SP 8450 UV detector set at a wavelength of 254 nm and a PC1000 software. The analytical column was a Hypersil® octadecylsilane (ODS) C18 reversed-phase column (15 cm x 4.6 mm, 5 µm particle size) (Thermo Electron Corp.) preceded by a Hypersil® ODS guard column (10 x 4.6 mm, Thermo Electron Corp.). The columns were maintained at 35°C. The mobile phase consisted of a gradient with solvent A (45 % methanol, 55 % H_2O, v/v) and solvent B (90 % methanol, 10 % H_2O), using a flow-rate of 1 ml/min. The gradient programme was initially 100 % solvent A, ramp to 35 % solvent B over 35 min and back to 100 % solvent A at 45 min. Total run time was 50 min**.**

The limits of detection (LOD) and quantification (LOQ) were 100 and 300 nM, respectively. The 6β-(OH)-testosterone metabolite was identified by comparison of retention time with an authentic standard from Steraloids (Newport, RI).

The cell protein content was determined with the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich).

Results

Cytotoxicity

The impact of active ingredients of herbal food supplements on cell proliferation and LDH leakage has been assessed on intestinal Caco-2 cells. LDH leakage measured after 6h and 24h exposure on differentiating (16 days post-confluence) cells did not reveal any cytotoxicity of pure compounds in the range of tested concentrations (Table 32).

On the contrary, a 48h exposure of proliferating cells with certain pure compounds at the highest concentrations tested had an effect on cell proliferation (31). Regarding St John's Wort, hypericin and hyperforin inhibited proliferation respectively from 10 and 1 ppm. In the group of terpene trilactones, typical compounds from ginkgo biloba, only ginkgolide J showed a slight inhibition at 25 ppm. The three flavonols tested inhibited proliferation from 10 ppm for isorhamnetin and kaempferol and at 25 ppm for quercetin dihydrate. Soy isoflavones revealed to be cytotoxic from 10 ppm for genistein and glycitein and from 1 ppm for daidzein. A very strong inhibition of proliferation was observed after exposure to 50 µM (D)L-sulforaphane, a typical compound of garlic.

Impact on CYP1A-dependent 7-ethoxyresorufin dealkylase (EROD) activity

CYP1A1 is an inducible cytochrome P-450 present in intestinal cells. Although most CYP oxidations are detoxification pathways, the chemistry of certain substrates leads to the production of reactive cytotoxic metabolites. For example, polycyclic aromatic hydrocarbons (PAHs), nitrosamines and food-derived aromatic amines may be activated to DNA-binding species by CYP1A.

A slight induction of CYP1A1 activity in Caco-2 cells has been measured after exposure to hypericin (6h and 24h), (-)-bilobalide (6h) and quercetin dihydrate (24h) from respectively 10 ppm, 50 ppm and 12,5 ppm (Table 33). Quercetin is yet known as a dietary ligand of the aryl hydrocarbon receptor (AhR), controlling CYP1A1 gene expression (Ciolino et al., 1999). Contradictory papers report induction or inhibition of CYP1A1 by extracts of ginkgo biloba (Yang et al., 2003, Oh and Chung, 2006, Chang et al., 2006). However, it is the first time that a terpene trilactone tested as a pure compound, proved to induce CYP1A1 activity.

When mimicking co-exposure in intestinal Caco-2 cells to pure compounds and B[a]P, a PAH typically inducing CYP1A1, the inhibition of B[a]P-induced CYP1A1 activity by certain compounds has been measured (Table 33). After co-exposure with hypericin, inhibition of B[a]P-induced CYP1A-dependent EROD activity was recorded at all concentrations tested. At 10 ppm, hyperforin showed strong inhibition. Polyphenols from both ginkgo biloba (isorhamnetin, kaempferol and quercetin dihydrate) and soy (genistein, daidzein and glycitein) revealed inhibition of CYP1A1 activity from respectively 1 and 12,5 ppm. Genistein and daidzein have yet been reported to inhibit CYP1A1-dependent EROD activity or gene expression in different models (Kim et al., 2000, Hukkanen et al., 2000, Shon et al., 2006, Helsby et al., 1998, Gradin et al., 1994). Inhibition has also been reported with isorhamnetin, kaempferol and quercetin at the level of CYP1A1 catalytic activity and/or gene expression (Kim et al., 2000, Pohl et al., 2006, Chaudhary and Willett, 2006, Kang et al.,

1999, Zhang et al., 2003). Regarding hypericin and hyperforin, major compounds of St John's Wort, Schwarz et al. (2003) reported inhibition of the formation of carcinogens by CYP1A1. As a consequence, these compounds may be considered as positive regarding detoxification of living organisms submitted to PAHs.

Impact on CYP3A4-dependent activity. Cytochrome P-450 3A4 is the predominant form in the intestine and contributes to the metabolism of approximately half the drugs in use today. Furthermore, it has also been proposed that small intestinal CYP3A4 could act in concert with P-glycoprotein to block systemic uptake of xenobiotics. A change in the metabolism of a drug by the co-administration of another substance is a frequent cause of clinically relevant drug interactions. In addition, it can also activate carcinogenic substances such as aflatoxins and PAHs. Different assays conducted with volunteers have shown significant induction of CYP3A4 activity after regular St John's Wort consumption (Durr et al., 2000, Wang et al., 2001). This inductive effect has been attributed to hyperforin. However, short-term consumption seems to have no effect on CYP3A4 activity (Wang et al., 2001).

In this assay, neither hypericin nor hyperforin inhibited vitamin D_3-induced CYP3A4 activity after 3h exposure (Figure 6). The induction of CYP3A4 activity has been tested after 2 weeks of exposure to hypericin and hyperforin. Only hyperforin induced CYP3A4 activity at 1 ppm, at a level almost as high as after exposure to 1α,25-$(OH)_2$-D_3, but the effect was not statistically significant (Figure 7). However, a treatment of two weeks with hyperforin 1 ppm seemed to be cytotoxic for cells, since the amount of proteins at the end of the assay dramatically decreased, making results hardly interpretable (results not shown).

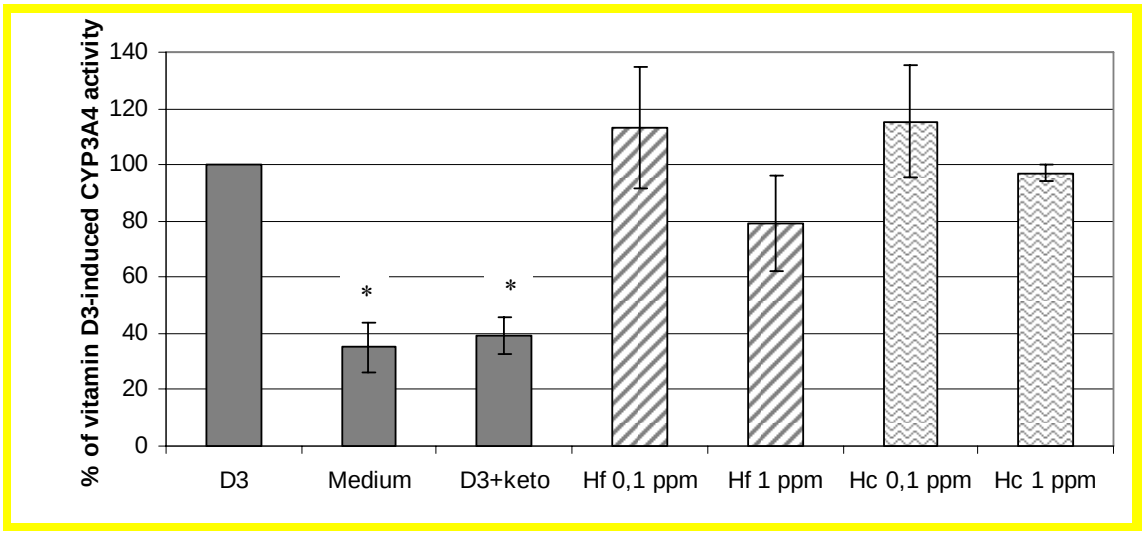


Figure 6: Inhibitory effect of hyperforine (Hf) and hypericin (Hc) on CYP3A4 activity. D3: maximal CYP3A4 activity after 2 weeks of treatment with 1α,25-$(OH)_2$-D_3 (100% = 4,56 pmol 6β-(0H)-testosterone.min^{-1}.mg $protein^{-1}$); Medium: basal CYP3A4 activity in cells without treatment; D3+keto: inhibition of 1α,25-$(OH)_2$-D_3-induced CYP3A4 activity by ketoconazole, a typical inhibitor. Results are expressed as % of 1α,25-$(OH)_2$-D_3-induced CYP3A4 activity and are means ± S.E.M. from 3 independent experiments (n = 9). * indicate $P < 0.05$ as compared to the control condition.

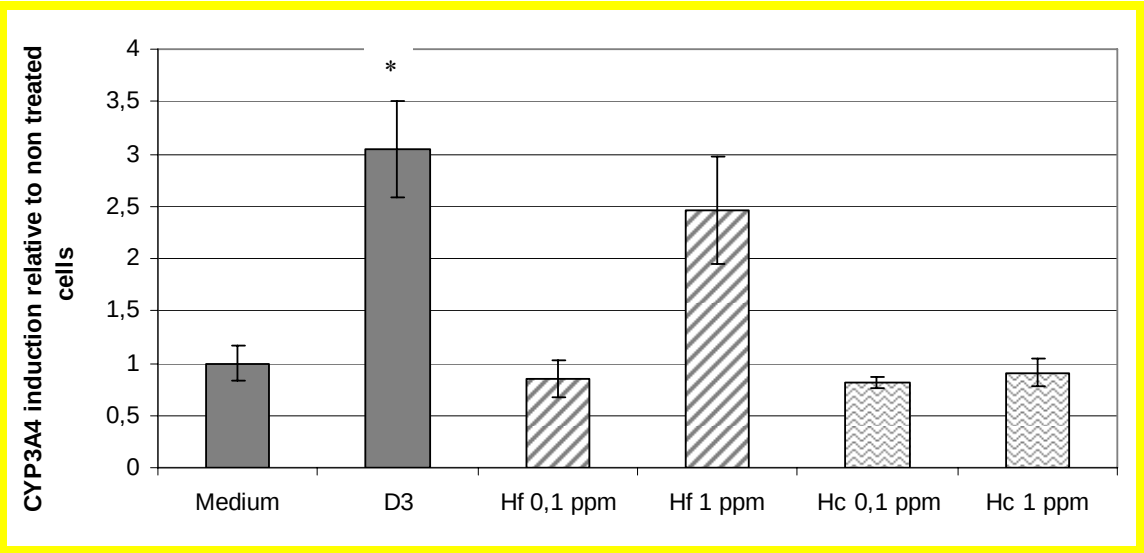


Figure 7: Inductive effect on CYP3A4 activity after 2 weeks of treatment with either 1α,25-(OH)2-D3 (D3), hyperforine (Hf) or hypericin (Hc). Medium: basal CYP3A4 activity in cells without treatment (1 = 1,5 pmol 6β-(0H)-testosterone.min-1.mg protein-1).
Results are expressed as CYP3A4 induction relative to non treated-cells and are means ± S.E.M. from 3 independent experiments (n = 9). * indicate $P < 0.05$ as compared to the control condition.

Table 32: Cytotoxic effects of pure compounds as standards on proliferating and differentiating Caco-2 cells. -: no effect; n.d.: not determined

Food supplement	***Compound***	***Cell proliferation (MTS assay)*** *48h exposure of proliferating cells*		***Cytotoxicity (LDH assay)*** *6h and 24h exposure of differentiating cells*	
		Concentration tested (ppm)	***Effect***	***Concentration tested (ppm)***	***Effect***
St john's wort	**Hypericin**	0,001 → 25	Inhibition from 10 ppm	-	-
	Hyperforin	0,001 → 10	Inhibition from 1 ppm	-	-
Ginkgo biloba	**Ginkgolide A**	0,01 → 25	-	1, 25 and 50	-
	Ginkgolide B	0,01 → 25	-	1, 25 and 50	-
	Ginkgolide C	0,01 → 25	-	1, 25 and 50	-
	Ginkgolide J	0,01 → 25	Slight inhibition at 25 ppm	1, 25 and 50	-
	(-)-Bilobalide	0,01 → 50	-	1, 50 and 100	-
	Isorhamnetin	0,01 → 50	Inhibition from 10 ppm	1, 12,5 and 25	-
	Kaempferol	0,01 → 25	Inhibition from 10 ppm	1, 12,5 and 25	-
	Quercetin.2H_2O	0,01 → 25	Slight inhibition at 25 ppm	1, 12,5 and 25	-
Soy isoflavones	**Genistein**	0,01 → 50	Inhibition from 10 ppm	1, 12,5 and 25	-
	Daidzein	0,01 → 50	Inhibition from 1 ppm	1, 12,5 and 25	-
	Glycitein	0,01 → 25	Slight inhibition from 10 ppm	1, 12,5 and 25	-
Garlic	**L-sulforaphane**	0,1 → 100 µM	Slight inhibition at 10 µM and strong inhibition from 50 µM	n.d.	n.d.
	DL-sulforaphane	0,1 → 100 µM	Slight inhibition at 10 µM and strong inhibition from 50 µM	n.d.	n.d.

Table 33: Impact of pure compounds as standards on (B[a]P-induced) CYP1A-dependent EROD activity in differentiating Caco-2 cells. -: no effect

Food supplement	***Compound***	***EROD assay***		
		Concentration tested (ppm)	***Impact on CYP1A-dependent activity*** *6h and 24h exposure of differentiating cells*	***Impact on B[a]P- induced CYP1A-dependent activity*** *6h exposure of differentiating cells*
St john's wort	**Hypericin**	0,001 → 10	Slight induction at 10 ppm (6h and 24h exposure)	Slight inhibition at all concentrations tested
	Hyperforin	0,001 → 10	-	Strong inhibition at 10 ppm
Ginkgo biloba	**Ginkgolide A**	1, 25 and 50	-	-
	Ginkgolide B	1, 25 and 50	-	-
	Ginkgolide C	1, 25 and 50	-	-
	Ginkgolide J	1, 25 and 50	-	-
	(-)-Bilobalide	1, 50 and 100	Slight induction from 50 ppm (6h exposure)	-
	Isorhamnetin	1, 12,5 and 25	-	Slight inhibition at 1 ppm and strong effect from 12,5 ppm
	Kaempferol	1, 12,5 and 25	-	Slight inhibition at 1 ppm and strong effect from 12,5 ppm
	Quercetin.2H_2O	1, 12,5 and 25	Slight induction from 12,5 ppm (24h exposure)	Slight inhibition at 1 ppm and strong effect from 12,5 ppm
Soy isoflavones	**Genistein**	1, 12,5 and 25	-	Strong inhibition from 12,5 ppm
	Daidzein	1, 12,5 and 25	-	Inhibition from 12,5 ppm
	Glycitein	1, 12,5 and 25	-	Inhibition from 12,5 ppm

Conclusions. We have shown that active compounds of herbal food supplements may be toxic and are able to modulate CYP1A1 activity in an *in vitro* model of the intestine, the first barrier encountered before entering systemic circulation. Since these herbal compounds reach intestinal cells at a possibly very high concentration in the same time as other chemicals like drugs, food contaminants or other food constituents, the consequences of such interactions should be taken into account. Some compounds, inhibiting B[a]P-induced CYP1A1 activity after co-exposure, could be considered as positive regarding detoxification of carcinogenic food contaminants such as PAHs. On the contrary, chemicals inducing CYP1A1 activity could participate to the conversion of pro-carcinogens to toxic DNA-binding metabolites, enven if detoxification mainly occurred with most chemicals. However, in this study, none of tested herbal compounds induced CYP1A1 activity at a level sufficiently high to be considered as a health issue.

3. CONCLUSIONS

In conclusion, from the food supplements samples we bought, it appears that a lot of them show chemical contaminations. It appears also that a lot of food supplements are sold on internet, generally without notification of the product.

However, notified, as well as non notified food supplements presented chemical contamination (heavy metals, and/or PAHs). Batch of food supplements non compliant for heavy metals have been recalled from the consumption market.

For mycotoxins, in 54 out of 61 food supplements, none of the 23 analyzed mycotoxins was detected. In the remaining 7 samples, levels found were low. There is no maximal level for mycotoxins in food supplements, but we detected OTA at a level above 2 µg/Kg (2.5 and 6 µg/Kg respectively), which is the European regulatory level for wine and grape juice. All the other detected mycotoxins were below maximal levels fixed for food.

For PAH, a preliminary risk assessment according 3 different approaches was performed. We showed that PAHs ingestion from the selected samples is not risky for human health, except in 1 or 5 cases, depending on the safety level considered.

Active ingredients to be assessed in *in vitro* assays have been selected and bought.

Active ingredients concentration in selected food supplements are currently analyzed in differents labs of the consortium.

First results indicate that active ingredient concentrations may vary a lot from samples to samples and clearly demonstrate the need of a control of the quality of the products available on the market.

The selected active ingredients are being analyzed *in vitro* and all results wil be completely interpreted when the calculation of working concentrations will be completed.

First results indicate a cytotoxicity of hyperforin and hypericin (active ingredient of St John's Wort) on both human HepG2 and Caco2 cell lines, at concentrations close to calculated plausible concentrations in body fluid after food supplement ingestion.

The final aim will be to check the cross reactivity of the contaminant and the active ingredient in *in vitro* assays. It will be important to analyze not only active ingredients as standards, but also samples extracts.

The survey of the food supplement consumption in Wallonia showed that food supplements are largely used, so the main recommendation at the moment is to be very careful in the communication of the results of this study. Indeed, it is clear that a lot of food supplements producers are making efforts (in the framework of the "autocontrôle" established in the new European and Belgian food legislation) to produce food supplements of high quality.

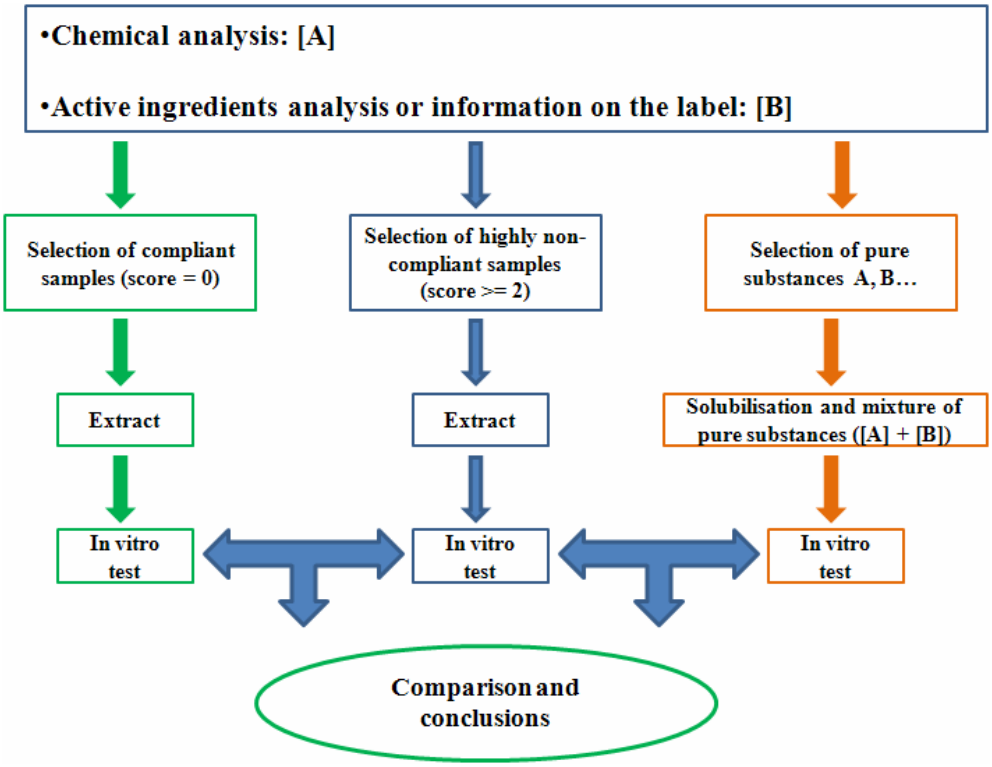
We will have to evidence problems of contamination linked to some food supplements products, but without giving to the public opinion the idea that all the food supplements are contaminated.

4. PERSPECTIVES FOR PHASE II

<u>Continuation of WP2 :</u> Biochemical and chemical analyses of active ingredients in food supplements.

The results of the first phase of this project show some interesting results on both toxicity and mode of action of the selected food supplements (see annex 7 for an overview of all the effects observed *in vitro* up to now). The challenges of the second phase will be to look at the interactions of the active ingredients of a FS at realistic concentrations and above all look at the interactions of FS with some of the found contaminants.

We plan for phase II to evaluate **mixtures of active ingredients and contaminants** on *in vitro* tests as schematized in the figure below :



As shown in this figure, the mixture of active ingredients and contaminants will be analyzed as follows: selection of highly non compliant and compliant food supplements will be performed. After that, based on the concentrations calculated from active ingredient and chemical analysis, solubilisation and mixtures will be performed. In parallel, an extraction procedure (using an *in vitro* digestion with enzymes) will be optimized to have a common extract for all the *in vitro* tests. The extract and the mixture of "pure substances" will be analyzed in parallel and compared to each other. This comparison will provide us information about the relevance of the *in vitro* test in regard of the lack of the matrice in the pure substances.

WP3 : risk assessment and communication

In this last part of the project, the relevancy of the *in vitro* results and the possible implications for the consumer's health will be assessed, in order to propose a screening of hazards and potential risks of the substances and mixtures tested, occurring in the human gut.

A second sociological part of the work is the presentation of scientific results to consumers, in order to determine if their risk perception has changed following scientific studies. Has the consumer confidence in food supplements change ? To realise this sociological study, a representative study case will be chosen.

Risk communication will be done at different levels:

- Level 1: Industrial stakeholders from food supplement industry. The communication will be set up via the user's committee.
- Level 2: consumers. The sociological part of this work concerning risk perception will allow us to establish a relationship with consumers. Risk will be communicated to them at the end of the study.
- Level 3: Public authorities. The final report will underline potential risks and will give recommendations for consumer's health. However, complementary studies will be necessary to have a global risk assessment.

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6. PUBLICATIONS

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