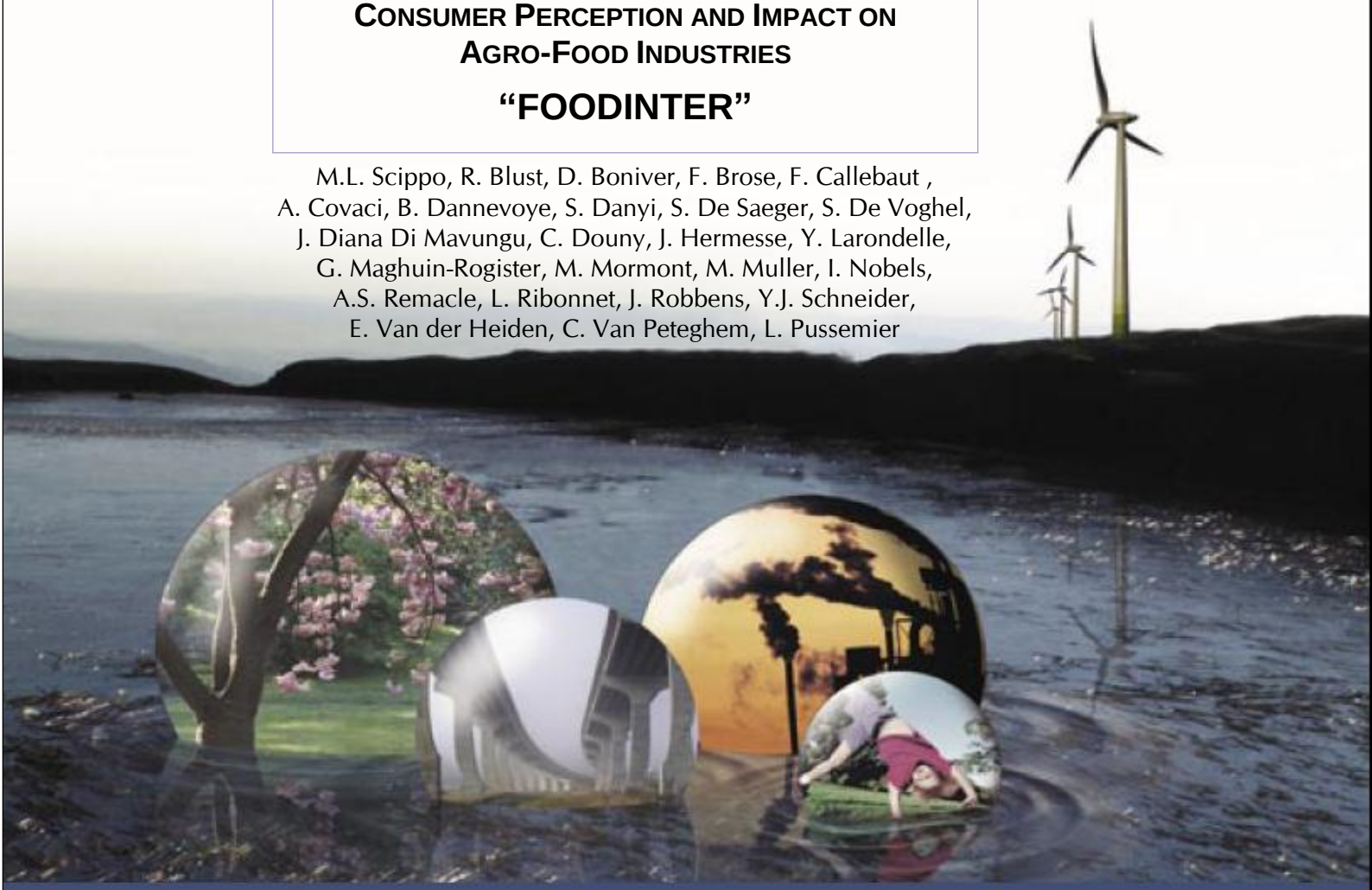
SSD

SCIENCE FOR A SUSTAINABLE DEVELOPMENT

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

"FOODINTER"

M.L. Scippo, R. Blust, D. Boniver, F. Brose, F. Callebaut ,
A. Covaci, B. Dannevoye, S. Danyi, S. De Saeger, S. De Voghel,
J. Diana Di Mavungu, C. Douny, J. Hermesse, Y. Larondelle,
G. Maghuin-Rogister, M. Mormont, M. Muller, I. Nobels,
A.S. Remacle, L. Ribonnet, J. Robbens, Y.J. Schneider,
E. Van der Heiden, C. Van Peteghem, L. Pussemier



ENERGY

TRANSPORT AND MOBILITY

AGRO-FOOD

HEALTH AND ENVIRONMENT

CLIMATE

BIODIVERSITY

ATMOSPHERE AND TERRESTRIAL AND MARINE ECOSYSTEMS

TRANSVERSAL ACTIONS

.be

SCIENCE FOR A SUSTAINABLE DEVELOPMENT
(SSD)

Agro-Food

FINAL REPORT

Food interactions : effects on health, consumer perception and impact on agro-food industries

"FOODINTER"

SD/AF/04

Promotors

Guy Maghuin-Rogister & Marie-Louise Scippo
Université de Liège (ULg)
Laboratory of Food Analysis Department of Food Sciences
Marc Mormont
Université de Liège (Ulg)
Unit of Socio-Economy, Environment and Development (SEED), Department of Environmental Sciences and Management,
Marc Muller
Université de Liège (Ulg)
Department of Life Sciences, Laboratory of Biology and Molecular Genetic, Faculty of Sciences,
Yvan Larondelle, Yves-Jacques Schneider
Université Catholique de Louvain (UCL)
Institut des Sciences de la Vie,
Luc Pussemier, Alfons Callebaut
Veterinary and Agrochemical Research Centre (CODA-CERVA-VAR)
Ronny Blust, Wim De Coen, Johan Robbens
Universiteit of Antwerpen (UA)
Laboratory of Ecophysiology, Biochemistry and Toxicology
Sarah De Saeger, Carlos Van Peteghem
Universiteit of Gent (UGent)
Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences

Authors

M.L. Scippo, F. Brose, B. Dannevoye , S. Danyi, C. Douny, J. Hermesse, G. Maghuin-Rogister, M. Mormont, M. Muller, A.S. Remacle & E. Van der Heiden (ULg)
Y. Larondelle, L. Ribonnet & Y.J. Schneider (UCL)
D. Boniver , F. Callebaut, S. De Voghel & L. Pussemier (CERVA-CODA)
R. Blust, A. Covaci, I. Nobels & J. Robbens (UA)
S. De Saeger, J. Diana Di Mavungu, C. Van Peteghem (UGent)

D/2011/1191/31
Published in 2011 by the Belgian Science Policy
Avenue Louise 231
Louizalaan 231
B-1050 Brussels
Belgium
Tel: +32 (0)2 238 34 11 – Fax: +32 (0)2 230 59 12
http://www.belspo.be

Contact person : : Christine Mathieu
+32 (0)2 238 34 93

M.L. Scippo, R. Blust, D. Boniver, F. Brose, F. Callebaut, A. Covaci, B. Dannevoye, S. Danyi, S. De Saeger, S. De Voghel, J. Diana Di Mavungu, C. Douny, J. Hermesse, Y. Larondelle, G. Maghuin-Rogister, M. Mormont, M. Muller, I. Nobels, A.S. Remacle, L. Ribonnet, J. Robbens, Y.J. Schneider, E. Van der Heiden, C. Van Peteghem, L. Pussemier. ***Food interactions : effects on health, consumer perception and impact on agro-food industries "FOODINTER".*** Final Report. Brussels : Belgian Science Policy 2011 – 143 p. (Research Programme Science for a Sustainable Development)

ACRONYMS AND ABBREVIATIONS

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
ANSES : Agence nationale de sécurité sanitaire (former AFSSA : Agence française pour la séxurité sanitaire des aliments)
As : Arsenic
Ba : Baryum
BaP : Benzo[a]pyrene
Bi : Bismuth
BEAU : Beauvericin
BMDL : Benchmark dose lower confidence limit
Bw : body weight
Cd : Cadmium
Co : Cobalt
Cr : Chromium
Cu : Copper
DAD : Diode array detection
DAS : Diacetoxyscirpenol
DHT : Dehydrotestosterone
DON : Deoxynivalenol
DRI : Dietary reference intake
EC : European community
EDI : Estimated daily intake
EMA : 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 Standards Agency
F-X : Fusarenon-X
GBE : *Gingko biloba* extract
GL : Glucosinolates
GRH : Glucoraphasatin
GTL : Glucotropaeolin
Hg : Mercury
HPLC/UV-FLD: High performance liquid chromatography/Ultra violet Fluorescence detection
HT-2 : HT-2 toxin
IAEA : Atomic Energy

IC50 :	Concentration at which 50% of the cell growth is inhibited
ICP-MS :	Inductively coupled plasma
ISO :	International Organisation for Standardisation
JECFA :	Joint FAO/WHO Experts Committee on Food Addi
LC50 :	Lethal concentration at which 50 % of the cells are dead
LC-MS :	Liquid chromatography tandem mass spectrometry
LOQ :	Limit of quantification
MB :	Moniteur belge
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 for standardization
NIV :	Nivalenol
OCP :	Organochlorine pesticide
OTA :	Ochratoxin A
PAH :	Polycyclic aromatic hydrocarbon
Pb :	Lead
PBDE :	Polybromodiphenylether
PC :	Plausible concentration
PCB :	Polychlorobiphenyls
PTWI :	Provisional tolerable weekly intake
RIVM :	National institute for public health (The Netherlands)
RP-LC :	reversed-phase liquid chromatography
RRF :	relative response factor
Sb :	Antimoine
SCF :	Scientific Committee for Food (European Commission)
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
WHO :	World Health Organization
ZEA :	zearalenone
Zn :	Zinc

Table of contents

SUMMARY

A. CONTEXT

Food supplements (FS) are subject to an ever growing interest, as they are now consumed by an increasing number of people. According to the results of a consumption survey conducted in France by the CCAF (2004), (N=1361), 11% of the grown-up respondents were FS consumers and 59% are consumers of « extended health food/products » (Gaigner, 2005). Botanical materials represent a large segment of this class of products (e.g. soy isoflavones, yam or hop extracts). The vast majority of plant based food supplements sold legally in the EU today is harmless under recommended conditions of use. However, plant extracts as such may not be harmless.

Furthermore severe cases of drug-plant extracts interactions have been well documented on patients (Abad, 2010, Chavez et al., 2005, Colalto, 2010, Izzo & Ernst, 2009), and it is likely that many more cases are not declared because not considered as originating from these interactions. Because of their vegetal origin and, in some cases, the non application of legal manufacturing guidelines (especially for materials prepared in third countries), the raw material used in preparing plant-based food supplements might also carry toxic environmental contaminants such as heavy metals or polycyclic aromatic hydrocarbons. It is important that plant-based FS comply with the legislation. Numerous legal requirements are in place in the EU to ascertain that food of vegetal origin does not contain potentially harmful amounts of residues or contaminants.

B. OBJECTIVES

The FOODINTER project aims to study selected food supplements (FS) sold on the Belgian market in terms of chemical contamination and the activity of their active ingredients. Six categories of plant derived products were chosen : gingko-biloba, St John's wort, soy isoflavones, maca, black radish and garlic.

The objective of this project is thus to contribute to the risk assessment of chemicals, natural compounds and environmental contaminants, present in FS which could interact between each other or with micro- or macronutrients of normal human diet. Interaction studies have been performed using existing *in vitro* models (based on various cultured cell types, prokaryotes and eukaryotes) with mixtures of active substances at concentrations that are very close to the real situation in human nutrition.

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. The objective is not only an educational planning, but also to promote a dialog between science and society in order to better identify the societal preoccupations and needs that research has to satisfy.

C. CONCLUSIONS

Our project was divided in 3 parts :

1. Selection and chemical analysis of samples of food supplements found on the Belgian market,
2. *In vitro* analysis of pure compounds identified as active ingredients of the selected food supplements, as well as food supplements extracts,
3. Study of the consumer perception of food supplements using the techniques of surveys, at the beginning of the project, and focus groups, at the beginning and the end of the project.

Selection and chemical analysis of samples of food supplements found on the Belgian market

We have selected for this project six different FS all made from one specific plant material:

- *Ginkgo biloba*: improve blood circulation and cerebral oxygenation
- St-John's wort (*Hypericum perforatum*): for mild depression
- Soy isoflavones (from *Glycine max*): reduce menopause effects
- Maca (*Lepidium meyenii*): increase libido and limit sexual disorders
- Black radish (*Raphanus sativus*): for bile secretion and intestine activity
- Garlic (*Allium sativum*): decrease arterial tension

In order to have an idea of what the consumer can find on the Belgian market, 61 samples were collected, purchased from 37 companies via internet (36 samples), pharmacies (18 samples) and specialized shops (7 samples). Twenty five FS were notified in Belgium whilst 36 were not notified (and generally available via the internet). This material was used to perform the analyses of chemical contaminants and active ingredients.

Analysis of chemical contaminants

Analysis of mineral éléments : 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 in force in 2007, at the time the samples were bought, for toxic element in FS (7 NC for Pb and 4 NC for Cd; one sample exceeded the maximal limit for both elements). Since 2008, the Belgian legislation has been replaced by a European Regulation, with higher maximal limits for Pb and Cd, and only 4 samples, ut of thèse 10, would remain NC for their Pb content.

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 maximal limit 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. However, in most cases, the PAH intake from FS is less than 5% the PAH intake from normal food.

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 detected 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					

For some of them, these active ingredients have been analyzed using chemical methods (HPLC or LC-MS) in order to determine the real content of the chosen FS in these active ingredients.

Analysis of six maca products (3 notified, 3 not notified) by nano-LC-MS revealed one product containing no lepidilin A and no lepidilin B. From these data, it could be concluded that this sample does not resemble a maca-extract. Nevertheless, macaridin was detected in all maca supplements, with no correlation with the presence of lepidilins.

The glucosinolate (GL) profile in black radish (*Raphanus sativus)* based dietary products was investigated. An analytical strategy combining the use of LC-PDA, LC-ESI-MS/MS and LC-APCI-MS/MS systems was applied. The LC-ESI-MS/MS system was used to detect and identify the naturally occurring intact GLs. The identified intact GLs were then desulfated and quantified on an LC-PDA system as desulfo-GLs. Prior to quantification, the desulfo-GLs were identified using an APCI-MS/MS system. In total, six glucosinolates were identified and determined (quantified) in the six analyzed products. The quantitative data revealed a great diversity in the individual GL which can be attributed to differences between species and subspecies of the different black radishes from which the products were derived. Different growing conditions could also have contributed to the differences in the glucosinolate content in the different samples.

According to the literature, glucoraphenin was found the most abundant glucosinolate in all samples.

The hypericin content specified on the packaging of thirteen different food supplements was registered. In five cases (not notified products), the announced content of hypericin leads to an intake higher than the legal limit of 700 mg/day in the "plant" Royal Decree of 1997 (MB, 1997).

We also observed the lack of hypericin content information on two notified food supplements.

The analysis of active ingredients in the selected products shows that the content of the product is not always mentioned on the label. This is mandatory, according to the European Directive 2002/46/CE, transposed in Belgian legislation in the "plant" Royal Decree of 1997. But, as this "plant" decree is not yet complete, because the active ingredients are not identified for each plant food supplement, this obligation is not applicable.

This shows the need for more research to identify active ingredients in plant FS as well as a gap to fill in the European and Belgian legislation.

In vitro analysis of pure compounds identified as active ingredients of the selected food supplements, as well as food supplements extracts

For the *in vitro* study, we focused on 3 categories of products, on the basis of the frequency of their consumption : soy isoflavones, St John Wort and *Ginkgo biloba*. For these 3 categories of products, we have tested their active ingredients separately with our panel of *in vitro* tests, as pure standards. The complete study (*in vitro* testing of active ingredients separately, mixture of active ingredients and plant extract) has been performed only on *Ginkgo biloba*, using a reference material (from the National Institute of Standardization, NIST), with certified concentration of active ingredients of *Ginkgo biloba*.

In order to work with plausible intestinal concentrations, we have estimated daily intake (EDI) of each selected compound, using FS content values either 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 to use as reference concentrations in our *in vitro* intestinal barrier model.

In all *in vitro* models, ranges of concentrations were tested, including the intestinal plausible concentration, as a reference.

The *in vitro* models used where :

- A model to study the general toxicity of the active ingredients in bacteria (*E.coli*) and eukaryotic cell lines (hepG2 and Caco2);
- A model to study the possible toxic mode of action of active ingredients using bacterial reporter gene assays allowing to investigate four mode of action : oxidative damage, DNA damage, general cell lesions and membrane damage;

- A model to study the possible endocrine disrupting and dioxin-like activity of active ingredients using eukaryotic reporter gene assays (where the interactions with respectively, human steroid receptors and rat and human aryl hydrocarbon receptors, were studied);
- A model to study the possible effects of active ingredients on human P450 (CYP) 1A1 activity in human colon adenocarcinoma cells (Caco2 cells).

The main results obtained with active ingredients at concentrations equal or below their calculated intestinal plausible level are indicated here below :

In **general toxicity assays**, some cytotoxicity was observed with hyperforin, bilobalide, isorhamnetin, kaempferol, genistein, daidzein and glycitein.

In **mode of actions (MOA) assays**, hypericin and hyperforin showed no effects, while an effect on DNA damage MOA was recorded for Gingkolide A, kaempferol and genistein, on cellular stress MOA for kampferol and on oxidative damage MOA for kaempferol, genistein and daidzein.

In the **Caco2 model to study the CYP1A1 activity**, hypericin was able to slightly induce the CYP1A1 activity as well as to inhibit the CYP1A1 induction in presence of BaP, while hyperforin was only able to slightly induce CYP1A1 activity.

The terpens from *Ginkgo biloba* (Gingkolides and bilobalide) as well as one flavonol (isorhamnetin) had no effect on the CYP1A1 activity, while the two other flavonols tested (kaempferol and quercetin) were able to induce as well as to inhibit the CYP1A1 activity. The whole *Ginkgo biloba* extract (NIST reference material) displayed the same kind of effect, but with a lower intensity.

Soy isoflavones (genistein, daidzein and glycitein) were able to inhibit CYP1A1, but not to activate it.

In the **study of the interaction with the aryl hydrocarbon receptor (AhR),** specific species effects were recorded. Both hypericin and hyperforin displayed the same effects : while they were unable to activate both the rat and human AhR, they potentiate the inductive effect of the reference ligand TCDD (tetrachlordibenzodioxin) in rat hepatoma cells (synergistic effect), but they inhibit this inductive effect in human hepatoma cells.

For terpens active ingredients of *Ginkgo biloba*, only Gingkolide J displayed an effect (activation of human AhR), and for flavonols, only quercetin was able to activate the AhR (in human but not rat hepatoma cells), while the three flavonolos (isorhamnetin, kaempferol and quercetin) inhibited the AhR (in both rat and human cells for isorhamnetin and quercetin and only in human cells for kaempferol).

The NIST *Ginkgo biloba* reference extract induced the AhR in both rat and human cells, acted in synergy with TCDD to induce AhR in rat cells (no inhibition

effect) and inhibit the TCDD induction of AhR in human cells. This last result is concordant with the observation made in Caco2 cells, where the NIST *Ginkgo biloba* extract both slightly induced the CYP1A1 (which is expressed in response of the activation of the Ah receptor, which is a transcription factor) and inhibited the CYP1A1 induction by BaP, another reference ligand for the Ah receptor.

For soy isoflavones (genistein, daidzein and glycitein), the results obtained when studying their effect on the Ah receptor are less concordant with the observations made on the CYP1A1 activity: the three isoflavones were able to induce the rat AhR but not the human, while genistein and daidzein acted synergically with TCDD to induce AhR in rat cells and only genistein inhibited the TCDD induction of AhR in human cells.

When studying the **possible endocrine disrupting activity of selected active ingredients**, hypericin and hyperforin were not able to activate steroid receptors (human estrogen, androgen, glucocorticoid and progesterone receptors), while hypericin inhibited the induction of the glucocorticoid receptor and both hypercin and hyperforin were able to inhibit the induction of the human estrogen receptor, showing a possible anti-estrogenic activity.

Terpens showed no activity on steroid receptors, while the 3 flavonols were able to induce the human estrogen receptor (showing an estrogenic activity) but also to inhibit the induction of the progesterone receptor (anti-progestagen activity).

The soy isoflavones, known to display an estrogenic activity, showed an expected activation effect on the estrogen receptor (showing that our model works), but also an inhibitory activity of the less studied progesterone and glucocorticoid receptors, showing a possible effect of these substance on other steroidal pathways than the estrogenic one.

To summary the first two parts of the project, the results show that potential risks, for public health, are linked to 3 major areas: interactions with drugs that may modulate their efficiency (shown from the literature study), environmental contaminants, such as heavy metals and polycyclic aromatic hydrocarbons (shown from the analysis of 61 samples bought on the Belgian market in 2007), and biological effects of certain active compounds that might for example act as endocrine disruptors for specific target groups or modulate the activity of the metabolizing enzyme CYP1A1.

Study of the consumer perception of food supplements using the techniques of surveys, at the beginning of the project, and focus groups, at the beginning and the end of the project.

From the consumers' encounter (third part of the project), we draw the following conclusions : the first is that **consumers do not exactly know what can be categorised as FS** and what can not. This raises the problem of a poor knowledge in the public of the exact definition and status of those products, even if paradoxically the categorization and definition of all health products seems heavily framed through regulation.

The second is that **FS (or products sold as FS) consumers are not a homogeneous group**; they have very different profiles and may share very few characteristics from one to another. Food supplements consumption is widespread among the population, but the monthly expense on those products generally ranges between 20-50€. The four main profiles we identified are: performance profile ; well-being profile ; deficiency profile ; prevention profile.

The third is that **consumers have generally low risk awareness** (even lower for interaction risks), lack a lot of information and of critical distance on those products, but want to be better informed. They also often make self-research on products, and can sometimes become hard to challenge (even by experts) as their main source of faith is themselves, the information they collected, their own experiences and feelings, or coming from trusted relatives. However, they can't be dismissed as "irrational" as they root their choices and judgements in personal history, values, "documented fears" (such as generalised low food quality), ...

The fourth is **that the status to give to consumers in face of risk management should be clarified and reflected on** ; indeed, consumers could play important roles regarding risk governance and should not simply be envisaged as passive receptors of information.

The fifth is that **strategies of risk management that only rely on control show their limits** in face of the new nature of risks, mainly complexity/systemic nature and uncertainty. Therefore, new strategies and new models of risk governance should be designed and experimented.

Several actions could be envisaged in order to decrease possible negative health impacts. For example, the implementation of the HACCP-guide for food supplements that has been developed by the Belgian food supplement sector and has been approved by the Belgian authorities (guide G-011 approved on 9/08/2007). This guide is a guideline for producers on how to implement a quality control system covering all the steps: from growing conditions through transport to preparation and conservation, using the HACCP approach (for Hazard Analysis Critical Control Point). This is already mandatory in Belgium, following the "Autocontrol" Royal Decree of November 2003 (Moniteur Belge, 2003), which is an implementation of the European Food Law (Regulation (EC) n° 178/2002). In this HACCP plan, **producers should reinforce their attention on chemical hazards**, such as heavy metals

(included at the moment in the European Legislation, but not when the project started), as well as polycyclic hydrocarbons (not yet included in the European Legislation). Targeted chemical controls might be envisaged depending on the plant species.

From the consumers' enquiry and the discussions in focus groups, we can make the following **recommendations** : the first is that the **communication strategy should take into account the diversity in consumer profiles and consumption patterns**. Therefore, a multifaceted communication strategy would be more suited than a too generalist and simplistic one.

The second is that an **Internet platform (assembling competent authorities, representative of industry, scientists and consumer's organizations) on risks associated with FS (and products sold as FS) consumption** would be a potentially very useful and powerful tool for consumers' information and empowerment, but also for risk governance, knowledge sharing with health professionals and mediators, and associated interdisciplinary research.

The third is that **health professionals (practitioners, specialists of nutrition, dieteticians, pharmacists, etc.) should follow more training.** Food supplements, alternative health products and nutrition (including problems of interactions between food and drugs) should be an essential part of their training. If those competences aren't mastered by practitioners (like patients complain about), then shouldn't the consultation of specialists of nutrition or FS be refunded by the healthcare system ?

The fourth is that **producers should give even more (and better) information on products and processes**, but also on theirs associated risks (including potential interactions with personal specificities, medicine and food).

The fifth is that **FS management and associated regulation, procedures or product categories should be, if not reworked, at least clarified for consumers** and allowing them to be more "responsible" actors in the risk management system.

D. CONTRIBUTION OF THE PROJECT IN A CONTEXT OF SCIENTIFIC SUPPORT TO A SUSTAINABLE DEVELOPMENT POLICY

This project would like to draw the attention of the authorities and all stakeholders on the risks linked to the free consumption of certain categories of food supplements, especially derived from plants.

All the recommendations described above (to improve : - quality control systems in FS production plants, - the information to the consumers, - the training of health professionals, - the communication between stakeholders) would result in a better management of public health in Belgium.

E. KEYWORDS

Food supplement, plant, *Ginkgo biloba*, St John's wort, Maca, Black radish, Garlic, Soy isoflavones, interaction, consumer, environmental, contaminant, heavy metal, mycotoxin, PAH, toxin, dioxin, hormone, herbal, natural, drug, cytochrome, stress, extract, active ingredient, labelling, notification

1 INTRODUCTION

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 (e.g. nutrients, vitamins, hormone-like substances, amino acids, anti-oxidants,...) and functional foods (e.g. phytosterols or omega-3 fatty acids enriched food) need attention as they are consumed by an increasing number of people. Food supplements do belong do to foodstuffs and are subjected to the European food law (EC, 2002), but the co-existence of botanical food supplements and herbal medicinal products, such as *Ginkgo biloba* or St John wort, which can belong to both categories, is confusing for the consumer.

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.

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

Interaction studies were performed using existing *in vitro* models (based on various cultured 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 was attempted. Nevertheless, it must be noted that n vitro studies only give an idea of possible interactions and have to be confirmed by *in vivo* studies.

From consumers surveys and discussion in focus groups, we also tried to analyze the place of food supplements in the diet and their impact on human health in order to increase the knowledge and fill some gaps regarding health claims and drawbacks that could be linked to these new habits in human nutrition.

Finally, the project also aimed at promoting better 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, the objective is also to promote a dialog between science and society in order to better identify the societal preoccupations and needs that research has to satisfy.

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

WP1. Preliminary information collection

WP2. Biochemical and chemical analyses of contaminants, active substances of food supplements

WP3. Risk assessment and communication, recommendations.

The results obtained from these 3 WP have been merged and are presented in the following section ("2. Methodology and results") in 7 major points :

2.1. Information collection and sociological investigation

2.2. Collection of samples, labelling and notification status

2.3. Analysis of environmental contaminants in selected food supplements

2.4. Analysis of active ingredients in selected food supplements

2.5. *In vitro* studies of pure active ingredients and food supplements extracts

2.6. Review of the literature about *in vitro* and *in vivo* effect of food supplement active ingredients

2.7. Risk assessment about selected food supplements categories

2 METHODOLOGY AND RESULTS

2.1 Information collection and sociological investigation about food supplements

The results that are presented here are a summary of the results we obtained through the six main "sociological" actions that we conducted in Foodinter (in both WP1 and WP3).

These tasks were :

(1) a review of European and Belgian legislation ;

(2) a review of social sciences literature dealing with risk (risk assessment, communication, management, …), consumer practices and representations about those products, marketing practices, etc. ;

(3) exploratory focus groups on FS consumers general concerns about those products ;

(4) surveys addressed to clients in FS retail places ;

(5) interviews with producers ;

(6) "risk focus groups" with consumers of FS.

Let's slightly detail the **methodology** of the tasks ranging from (3) to (6) :

(3) The main objectives of exploratory focus groups were to examine social representations of FS, and opinions consumers wanted to express. We organized three focus groups, intended for both consumers and non-consumers, and carried out in three meetings of two hours each. The number of participants varied between 6 and 12. Four outside participants also contributed as experts to these discussions through presentations. The groups were heterogeneous in terms of age, social situation but most of the participants were woman more or less interested in the question. Within both the interviewees and the focus groups participants, both working class strata and men were under represented categories, probably for cultural reasons that go beyond the scope of this research. This is an important point to consider, however, when discussing communication strategies from the results on.

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 on 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.

(4) Two identical surveys were then conducted by the teams of ULg (Socio Economy Environment and Development, Marc Mormont and co-workers) and CERVA-CODA (Luc Pussemier and co-workers). They consisted in 20 questions, both open and closed, addressed to clients in FS retail places.

The first survey took place in Liège (167 respondents), addressed clients of drugstores, health food shops and organic food stores, herbalists, and supermarkets; the second was conducted in Ghent (276 respondents)[1], and addressed only to clients of pharmacies.

The objectives of these questionnaires were to get a better understanding of : (a) knowledge and perceptions of food supplements, (b) consumption practices and frequency of FS consumption, (c) the budget allocated to their consumption, and (d) the perception of possible risks (See questionnaire in annex of the final report of phase I of the present project in Mormont et al., 2009). The survey took place on food supplements retail outlets and was addressed more specifically to FS consumers ; it consequently induced a positive effect on the mean knowledge of respondents about those products, as well as the percentage of FS consumers among them.

(5) Consultation of FS producers has been made through individual interviews with company officers. Four different producers' were interviewed (semi-directive interviews) to explore the way producers manage the risk aspects of food in this specific context.

The objectives of these interviews were to grasp : (a) the level of information companies have about contaminants and problems of possible interactions, as well as the importance of "interaction risks" or "systemic risks" in the firms' research activities ; (b) the importance of food safety in the company's strategies or research pools (traceability systems, contaminants and interaction-related risk management systems, opinions on product regulation, …) ; (c) the place of consumer's preoccupations and practices in this strategy.

(6) Two sessions of three hours each were organized in Liège, each with the same group of participants (9 people). They were all FS consumers, and wanted to know more about FS or give their opinion. The proposed approach doesn't aim at representing exhaustively citizens' opinions, but rather at exploring what would be an informed citizen's or consumer's framing of the central questions behind the Foodinter project, that is in general risk issues regarding FS. The process was the following :

1 The Ghent survey was performed by students of the Faculty of pharmacy of the University of Ghent, under the supervision of Sarah Desaeger and Carlos Van Peteghem.

Sequence 1 : citizens are called to express their preoccupations about food safety and risks in FS production, marketing or consumption.

Sequence 2 : citizens are provided with scientific information on the results of the FOODINTER research ; they are invited to formulate any questions or remarks.

Sequence 3 : citizens are slightly provided with information, web links to food-chain security or risk management agencies, and collective reflexions on what are risk communication actions stakeholders implement nowadays about FS (consumer associations, industry, health professionals, etc), what could be their practices and strategies or attitudes towards risk.

Sequence 4 : (4a) Citizens are called to formulate remarks or concerns regarding risks associated with FS, discuss those remarks altogether, and then (4b) formulate remarks, proposals or recommendations on the communication of the results, as well as extensively on general risk communication, and/or risk management regarding FS.

This process induced a "progressive informed framing" that helped researchers to shape the scientific recommendations on risk communication.

2.1.1 Legislation review

European regulations

On European level, food supplements are regulated by the directive 2002/46/EC. This directive is a first step towards harmonization of the different national legislations.

Therefore this Directive helps gathering better conditions for free circulation of FS, equal competition conditions in Europe, and protection of consumers. Indeed, each country has its own regulation or notification scheme regarding food supplements, which can show differences (though they tend to decrease with 2002/46/EC, as European directives have to be transposed in national legislation).

This directive defined 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, multi-vitamin and multi-mineral tablets or capsules or capsules of plant extracts such as valerian, garlic,…

It results from this definition that FS are foods and therefore are also the subject of the Regulation (EC) 178/2002 of the European Parliament and of the Council of 28th January 2002, establishes the **general principles and prescriptions of food legislation**, instituting the European food security Authority and determining procedures for foodstuff safety. This regulation is the basis of food safety regulation at European and national levels, as is directly applicable. In the definition of food in this regulation, medical products are excluded. Consequently the Directive 2002/46/EC doesn't apply to medicinal products defined in Directive 2001/83/EC (EC, 2001), enforcing a communitarian code for medicinal products for human use nor to **traditional herbal medicinal products** as defined in Directive (EC) N° 2004/24/EC of the European Parliament and of the Council of 31 March 2004 amends Directive 2001/83/EC on the Community code relating to medicinal products for human use (EC, 2001).

Regulation N° 852/2004 deals with food hygiene.

General **labelling** provisions and definitions are contained in Directive 2000/13/EC (EC, 2000).

European Regulation (EC) N° 1924/2006 concerns **nutrition and health claims** made on foods, that applies to FS as they *are* food according to the regulator.

The Confederation of the Food and Drink Industries of the EU (CIAA), European Responsible Nutrition Alliance (ERNA), European Federation of Health Products Manufacturers (EHPM) and European Botanical Forum (EBF) have jointly contributed to the « Food industry's contribution to the list of claims according to Article 13 of the regulation 1924/2006 » which aims at compiling a list of health relationships for nutrients or substances to be evaluated by EFSA in accordance with Article 13 of this Regulation, with corresponding scientific references. It can be considered as the first part of the claims that have been submitted for evaluation in the so-called "Article 13 list". It covers several sections: vitamins, minerals, carbohydrates, protein, fats, fiber, probiotics, foods and beverages, diets, other substances and botanicals.

We could underline that this regulation has made emerge critics and blur on its application. According to the CIAA (Confederation of the Food and Drink Industries), and despite the consequent work undertaken by the European Food Safety Agency (EFSA) on regulation 1924/2006 guidance (EFSA, 2010), *« there is still much uncertainty as to what is required by way of the scientific substantiation of such claims. The consequence of this is that there is still insufficient clarity for industry applicants and a need, therefore, to re-examine the process for dealing with claims in this and other areas of new and emerging science »* (CIAA, 2010).

The European Commission announced in a communication (27/09/2010) (Europa, 2010) the delaying of health claims regarding « botanicals » from the procedure of the progressive review by EFSA of the huge quantity of health claims

known as « Article 13 ». The reasons are insufficient time to evaluate all claims (more than 5000!), but also divergences in opinions and conflicts about the way plants are « treated » in the regulation, and in the « Traditional Herbal Medicine Products Directive » (THMPD) that have to be resolved first.

Regulations (EC) N° 396/2005 and 1881/2006 of the European Commission of 19 December 2006, sets maximum levels of pesticides and of certain **contaminants** in foodstuff respectively.

Finally, let's quote Regulation (EC) N° 1925/2006 (EC, 2006) concerning the addition of vitamins, minerals and some other substances to foodstuff.

Obviously, all horizontal and vertical legislation applying to food or to specific substances also applies to food supplements when justified.

We can remark in this review cases of the European Court of Justice that show that the legislation surrounding "new health products" and especially « foood supplements », often qualified as "border" products, can be subject to divergent interpretations (see for instance cases C-140/07 and C-88/07).

Belgian regulations : In Belgium, there a three Royal Decrees, and two Ministerial Orders, that apply to food supplements and that have been modified in order to transpose the European directive 2002/46/EC.

In the three Royal Decrees, 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.

These Royal Decrees mention the notification process through which a FS has to go in order to be marketed in Belgium. There are indeed three relatively similar notification processes for the three categories of products created through law (Nutrients (NUT), Plants (PL) and Other Substances (AS)) covered by each of the three Royal Decrees described here (the notification process will be detailed below).

In these three Decrees, it is mentioned that "*in the labeling, displaying and advertising for food supplements, it is banned : 1° to give the product preventive, curative or therapeutic properties or evoke similar properties ; and 2° to state or suggest that a balanced and diversified diet is not a sufficient source of Nutrients in general".*

The first Royal Decree tackles the issue of Nutrients and their use into food supplements (AR 3/03/92). The first Ministerial Order (AM 21/05/2003) (Moniteur

Belge, 2003) relates to AR/3/03/92 and determines which are the chemical forms of vitamins and minerals that can be used in FS.

The second Royal Decree (AR 29/08/1997) concerns plants and plant preparations. In the appendix to this decree, there are three lists:

(1) a list of dangerous plants whose use for direct consumption or as ingredient of preparation is strictly prohibited.

(2) a list of eatable mushrooms.

(3) a list of plants that may be used in food supplements which have to be notified. For some of those plants, maximum amounts are laid down per daily portion, for which a list of recommended analysis methods has been drawn up

The third Royal Decree (AR 12/02/2009) (Moniteur Belge, 2009a) regards manufacturing and marketing of food supplements containing substances other than nutrients and plants or plant preparations.

The second Ministerial Order (AM 19/02/2009) (Moniteur Belge, 2009b) relates to AR 12/02/2009 and also regards manufacturing and marketing of food supplements containing substances other than Nutrients and plants or plant preparations.

The label of FS shall bear all mandatory indications, as for ordinary foods, as described in the royal decree of 13/09/1999 (Moniteur Belge, 1999) about pre-packed foodstuff labelling. Besides this, the label of food supplements shall bear a series of additional indications:

(1) the name "food supplement";
(2) the recommended daily intake (RDI);
(3) a warning not to exceed the recommended daily intake;
(4) a statement that the products should be stored out of the reach of young children;
(5) a statement that food supplements should not be used as a substitute for a varied diet;
(6) the amounts of nutrients present in the product per recommended daily portion (this may also be given in graphical form);
(7) the name of the plant(s) in the language of the region (when available), as well as the scientific name (for food supplements containing plants).

During the notification process, every notification file (of products falling in one of the three categories cited above (NUT ; PL ; AS)) is examined by the Belgian authorities (Federal Public Service "Health, Food Chain Safety and Environment").

The notification file shall contain, among others, the following items :

(1) the nature of the product;
(2) the complete list of the ingredients of the product (qualitative and quantitative);

(3) if applicable, the nutritional composition (or analysis) of the product ;
(4) the labelling of the product;
(5) data required to appreciate the nutritional value of the product;
(6) the commitment of producers to realize frequent analysis of the product, at various moments, and to let the results at the availability of the Service;
(7) the evidence of payment of a fee to the public authorities for every notified product.

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.

In case of any breach of the foodstuffs legislation, the product will not receive a notification number (NUT), will not be allowed on the market. These products will be named in this report "non-notified product" to distinguish it from notified food supplements. Examples of a breach of the legislation are: excessive maximum amounts, too high doses or use of prohibited additives. All notified products are stated in a list updated regularly, published on Federal Public Service "Health, Food Chain Safety and Environment" website :
(http://www.health.belgium.be/eportal/foodsafety/foodstuffs/foodsupplements/index.htm?fodnlang=fr)

The Superior Health council (SHC) and the Commission for advice on plant preparations play an important role in the legislation process. The SHC expresses recommendation on specific matters (such as lately, recommendations on maximum concentration of lycopene and luteine in FS), and on general Belgian health and food security.

The commission for advice on plant preparations is responsible , among other, for updating the lists of forbidden and permitted plants, the determination which parts of plants can or cannot be used, the determination of maximal dosages,….

Both these scientific organizations can also be asked for advice for specific dossiers when the applicant asks for a derogation of the legislation (for example to exceed the maximum levels of nutrients or to use non-harmful plant preparations from plants that are mentioned on the first list in the annex of the decree on plants).

The report "Nutritional recommendations for Belgium 2009" (SHC-8309), mentions that FS and FF can be used to compensate for deficiencies, or in particular physio-pathological situations, what indeed corresponds to only a small part of FS consumers as we will see.

For another version of legislation review, the European Botanical Forum published a guiding document. This can be downloaded from EBF web site :
http://www.botanicalforum.eu/uploads/ebf_factsheets.pdf

2.1.2 Review of the social sciences literature on risk communication, risk management, and food supplements consumers' practices or representations about those products

We summarized this review into three main study fields :

A first study field investigates the consumption practices of FS consumers, as well as their representations and "needs" or demands. Example in this field are : the BfR (German Federal Institute for Risk Assessment) research project aiming at understanding which information do consumers need about FS, entitled "*Target group-driven risk communication on food supplements*" (BfR, 2008).

We can also cite the work of Touvier & al. (2003), or of Gaigner and Hebel (2005), trying to know who are the consumers of FS and understand consumption practices.

In this field, we could also underline studies on the evolution of consumers' relation with, definition or perception of "risk". Let's remark that we can find in studies a tendency to split, on one side "citizen's views", that are often tainted with fear or irrationality, and on the other "experts' views", that are presented as rational and even uncontroversial.

A second study field aims at understanding the evolving nature of risks along with "modernity" and the way risk communication, management (through policy, or governance) should adapt to this deep shift. This field can also examine food "quality", standards, control and certification processes, as well as their evolution.

A first example is to be found through the interesting work of Beck G. and Kropp (2010), who analysed the problems and failures in conventional expert-based risk communication in face of uncertainty, precisely in the case of FS ; their study, appealing for new management frames and practices, is based on Ulrich Beck's theoretical frame of "risk society" or "risks of Second Modernity" (Beck, 1986 ; 1991).

Renn and Klinke (2004) presented new concepts for risk management and communication, in face of new, complex and "systemic" risks.

Let's also cite Brown (2009), who explores the evolution of the models of risk communication. First step is a well-spread "deficit model" in which consumers' "bad" practices or "misunderstandings" should be fixed through expert-based assessment and advice. The second step is a "new deficit model", in which science, asked to remove all current uncertainties, gets "paralysed" by the need of analysing everything, what is hardly feasible in a context of complexity. The more you know, the more you realize that you need additional information. Pace of evolution is quick

(with new products or processes), and finally uncertainty is (and will probably always be) a fundamental compound of risk. "*Although an enormous amount of research is needed, I believe that the argument for more research (…) provides an illusion that the deficit can be fixed (…) [and] fails to deal with the real issue, which is how to regulate in face of uncertainty*" (Brown, 2009). Brown tries then to describe an ideal, adaptive governance regime, where these failures can be addressed, mainly by being more transparent for consumers and allowing two-sided transmission, cooperation and reflexivity between the three pillars or risk governance (namely risk assessment, risk communication and risk management). This governance regime would have the four following characteristics: (1) informed for "governors" ; (2) transparent for consumers ; (3) prospective (as opposed to reactive), providing mechanisms to anticipate future, yet unknown harm ; and (4) adaptive and reflexive (as it can never be finished nor perfect, but has to be continuously built "on the move", from its experiences).

In this field, we could also cite the work of Callon, Latour, Lascoumes and Barthe (2001) around the question of "how to act in uncertainty ?".

Finally, the third study field we identified aims at understanding the evolution of the roles and missions of science in face of risk in this "context", as well as the potential of development of new, interesting collaboration between disciplines or between "social categories" : between professionals, researchers and experts, policy, agencies, as well as citizens or organised consumers.

Let's here cite Weingart (2004) or Gago (2004), who details the evolution of scientific activity in face of the challenges of public trust and risk governance.

2.1.3 Summary of the results obtained through the surveys and "risk focus groups" with food supplements consumers2

- **Unclear definition of "food supplements" and low understanding of the management system of food supplements**

Respondents to surveys do not exactly know what kind of preparations can be categorized as food supplements, showing for example a lot of hesitation for vitamins and plant extracts or oils.

This is to be linked with the "blurry" and hardly shared status of FS – be it among consumers or between them and the law, networks of scientists or experts, professionals, … . This status is see-sawing between food and medicine, and makes the "category" of FS appear as a very heterogeneous one, even for some a "non-category" or a marketing invention (as the products sold under this appellation

2 The detailed results of the survey (including the questionnaire) and focus groups can be found in a separate and comprehensive publication, is available on demand or will be published on the website of the project.

existed far before their large scale marketing, and often in different forms or processed differently).

So do FS give the beneficial effects of both medicinal products and food without being any, *stricto sensu* ? Moreover, how could the product be more precisely defined than by a literal definition, such as "FS complete nutrition", what indeed doesn't mean anything as we are supposed to feed "well". In the same sense, to what products sends the expression "alternative health products"? We could observe that it's impossible to have clear, consensual answers to these questions.

This « blurry vision » seems to be exacerbated as a lot of actors, from the producers to the private, family-member or relative adviser perpetrate this blur and "convince" with arguments crossing the fields of prevention, treatment, performances or well-being.

The way that regulation and administration have chosen is to try to stabilize categories, and to examine each product in turn. Products are to be sorted in "FS" category (regulated as food) or medicinal products ; but this seems very complex[3] and unknown or misunderstood by the public ! Consumers didn't understand well the categories of law ("medicinal product", "food supplement", "herbal medicinal product", "other", ...) and what they trustfully assess, while the same seems true for quality controls. This unveils the important question of the trust of consumers in production and risk management actors, as well as in the risk management system and procedures themselves. We can see the risk of a gap growing between consumers and all other actors of products or risk management if trust would come to diminish again.

It is hard to believe that consumers frame or define these "categories" of products similarly than regulation, when stating for instance that *"food is the first medicine".* They then surely don't frame similarly the interconnections of these categories following their naming : if we eat always properly, will we need medicine or FS anymore ? This raises also consumers' concerns about their feeling of a globally degrading quality of food in "modern" societies, that they fail to address when compensating with FS...but which is at the same time a potentially big motivation for other consumers to take FS !

Indeed, for consumers, FS (or assimilated products) consumption isn't rooted in a « cold and closed definition », and is neither a mechanical act, but a living, a personal experience, rooted in their history, habits, thoughts, representations and values, and mixing the field of food or nutrition with the one of medicine or medical treatment. Food and medicine are possessed by a symbolic dimension that shouldn't be underestimated when assessing social representations of FS. Food and medicine are two very different pools of images and representations that are both activated and mixed in complex, sometimes paradoxical ways when consumers are put in front

3 See for instance cases of the European Court of Justice C-140/07 and C-88/07.

of FS (and the "wave" of all other "health products"). This underlines the potential of those products to make consumers "loose their marks", failing to put their consumption of FS in perspective.

- **A high heterogeneity in consumer "profiles" and consumption patterns**

There is no "mean consumer" of FS, such as there is no "typical" FS but a wide range of products and applications.

This heterogeneity can be detailed though the following dimensions : we could first observe a high heterogeneity in the motivations or in the objectives consumers pursue through FS consumption (see Figure 1). This was noticeable through the surveys, and was verified through focus groups with consumers, though these profiles should be refined (and eventually new ones added) with a more detailed and representative survey. This first level of heterogeneity is according to us the most prominent to understand consumers' practices and to design a suited communication strategy. Thus, other levels of heterogeneity detailed afterwards should be linked to this first heterogeneity in consumer profiles or consumption patterns.

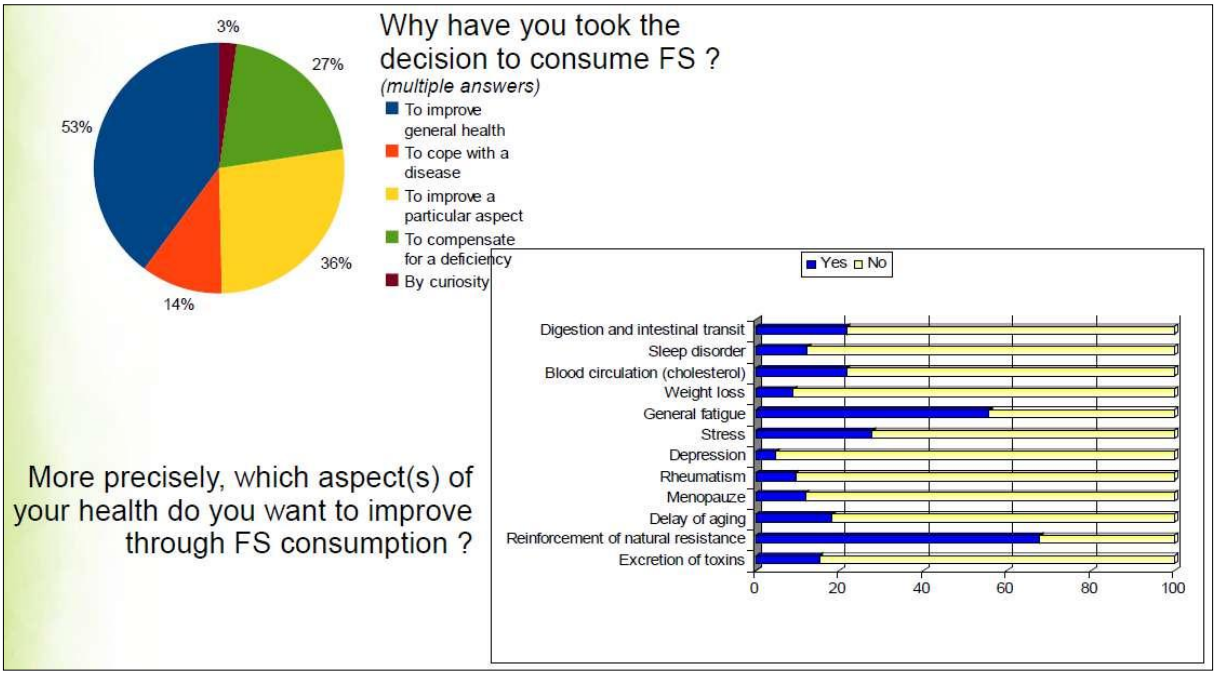


Figure 1 : Consumers' motivations to consume FS.

REM : In this graph and following, percentages are means of the two surveys, and overtake 100% since multiple answers were allowed.

This diversity (and diversity in the products used) can also be based upon gender : women seem to be relatively more interested in « well-being », alternative health and therapies, or diet ; male consumers seem from their side to be more interested in the boosting of performances (especially true for sport or fitness). But it depends of course of a lot of other factors, such as age or health situation (if one has

chronicle diseases, deficiencies, etc.), and upon other « subjective criteria » such as the degree of conviction in the products used and its effects, the mode of relation to a product regarded as « natural », the origin of the decision to consume FS (see Figure 2), the values, knowledge or tips transmitted from relatives (as well as practitioners, articles, ...),...

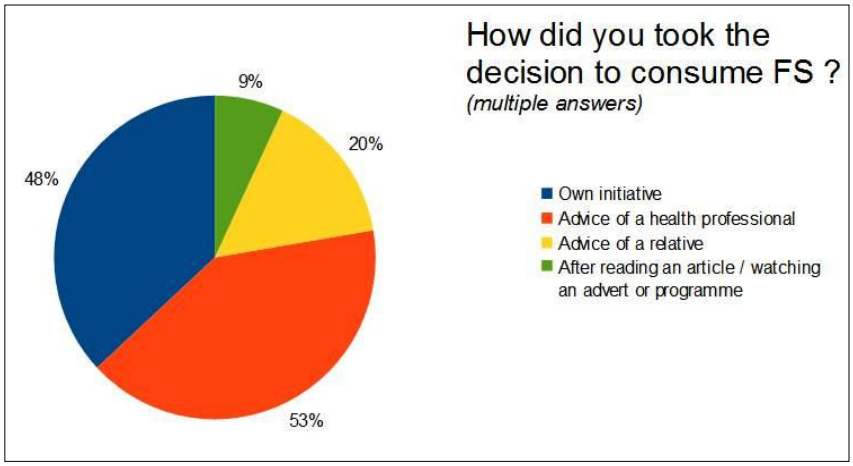


Figure 2 : How are influenced consumers' decision to consume FS ?

We identified four main profiles or patterns , drawn as ideal-types :

"Performance" profile (sport, studying/working), where FS consumption is motivated by (or "makes possible") the improvement of one's physical or mental capacities and performance. Performance seems rooted in a kind of functional "problem-solution" approach (the problem being to be not powerful enough, or to perceive that its physical or mental limits are too low compared to what is expected or "possible" thanks to new substances).

We could also widen the range of this "*performance*" category to include products used to *improve* anything, be it appearance, aesthetic, outline, hair or nails resistance, etc.

The link to "natural" or "health" seems to be the weakest in this profile, but would tends to reinforce when including aesthetic or outline purposes.

"Well-being" profile (or "smartening up", "healthy life", ... profiles), where FS consumption is motivated by the reaching of a balanced diet, long-lasting and "healthy" life.

A first problem here is to acknowledge on the impossibility to define these qualificatives ("healthy", balanced", ...) (1) differently than by the negative ("the absence of troubles") and (2) in an objective, measurable way (as troubles are always "perceived" and are to be assessed through medical assessment). The

problem here is then the absence of marks by which a state of "good health" could be judged by consumers, who bare the diffuse risk not to be able to escape from a too narrow, subjective and idealistic view about their state of health, or in other words, to engage an "endless quest".

In this profile, "natural" qualities (though unclearly defined or assessed) of the product are central for consumers and even makes FS prevail on conventional medicine, which seems often perceived as very criticized and untrusted "chemical crap", that they want to avoid as much as possible. This relation to the "natural" in this profile is also central as unhealthy or "unnatural" diets and *"modern lifestyles [are] threatening and should be challenged"*. This appears rather paradoxical, as for other consumers, FS consumption isn't considered as a solution as it can inherently give breath to "unnecessary" health products consumption, containing the risk to ingest "(chemical) crap" as well, when the look for a balanced and healthier food would be required at the root. But FS, we can say, hold more promises than "normal food", may it be healthy, organic one : they are sold as acting quickly, being relatively cheap, not very harmful (thought sometime encouraged by relatives, articles, or the for the "reason" that FS are sold over-the-counter and can allow "self-treatment"), etc. … arguments that some consumers would want to be banned or dismantled by public health authorities as it stimulates FS consumption, and particularly unnecessary FS consumption.

For important this challenge of "having a healthy life" can be nowadays (what we don't judge here), we can ask where would the limits be, talking about ideals such as "well-being" or "harmony" ? How could they be defined ? How could this reasoning be empowered, be put in perspective in face of manipulation or propaganda risks from the industry, letting this ideal or "purity" model be pushed too far in order to boost consumption ?

Deficiency profile ; consumers are here more "forced to" take FS, as they may have a chronic illness (for instance digestion troubles) or particular deficiencies (for instance a mineral deficiency or temporary blood circulation troubles). We could therefore draw two sub-categories in the deficiency profile, or more likely continuum based on the length or frequency of the treatment, and its character of necessity towards the trouble.

The "curative", "problem-solving" dimension in this consumption profile is central, and could obviously hardly be addressed in the same manner the "performance" or "well-being" categories. "Problems" have to be objectified through medical assessment. What will also differ from those profiles is that this call for performance or "well-being" is virtually unlimited, and comes from a mix of psychological and social pressures, from society's increasing pace as well as

from one's personal wills, myths and "dreams" about his body (pushing the limits further, reach "physical harmony", cope with tiredness, ...).

We could add that it can be sometimes very difficult to judge if one's troubles are "real" or perceived/exaggerated. They could also be caused by multiple and sustained auto-treatments consumers can do on their own, as some could also be some kind of "hypochondriac" ; in a lot of cases, it's also hard to say if the perceived "treatment" doesn't give breath to problems, or at least to their ongoing perception. Anyway, our role couldn't be to judge consumers on this very sensitive issue, and this would be more dependent on the competence of doctors and nutrition specialists. But we could also make the hypothesis, comforted by consumer's sharing of experiences, that practitioners lack competences, professional conscience, or simply time to overcome this task (such as interdisciplinary, long-term, complex and deep studies or analysis about nutrition and practices, in relation with mind and thoughts, patient's relation to illness, …).

We could rise the question of competition between cheap, but poor advice everyone can find on internet, and costly (to very costly for specialists) but good advice one has to ask his practitioner (and engage those complex, expensive, long-term analysis). Moreover, it can be *sometimes very difficult to find a "good" practitioner, one who "gives* real*, useful tips*", "*is objective and doesn't look to manipulate you*", or simply "*one who listens to you*", "*one that takes the time to (…)*".

Relation to the "natural" could also be important in the deficiency profile, but it's not very clear here (depends on consumer's trajectory).

Prevention profile ; typical examples would be the autumn vitamins and minerals treatment, or omega-3 and -6 fatty acids consumption. We have to warn that it can be sometimes hard to distinguish between "performance" and "prevention" profiles, as they may both be rooted in the same "improvement" logic, that insists for instance on the strengthening of natural defences and of "tonus" as well to remain healthy.

It's important to underline that this pattern is the more widespread among FS consumers, as shown through the surveys (about half of the respondents). They want to reinforce their immune system and fight against tiredness (what obviously corresponds to the widespread vitamins and mineral cures) and stress. This consumption is recommended by practitioners for a long time on, and is rooted in "traditional" or "familial" medical practices.

This heterogeneity is connected to networks of advice, and of advisers or "mediators", that are people or information sources that influence FS consumption : they can be formal networks, such as for practitioners or specialists, but also more

informal such for web sites (often partial or uncontrolled), but also sport trainers or "natural therapists", friends or relatives and their experiences ("uniniated" knowledge), …

Labels and description of products are also important sources of information, as most of the interviewed consumers read labels and are convinced of the beneficial effects of the products as producers describe them.

We can also point out various levels of information of consumers (or of "access to information") ; some are real "information-hunter" (and deplore huge lacks in "good information), while others will never look for any. This is also to be linked with different degrees in perception of risk by consumers.

A large part of questioned people (around 50%) do consume food supplements from their own initiative (without any medical advice), while around 50% took the decision on medical advice and 30% following relatives' advices (multiple answers). Consumers don't share the same relation with their practitioner or specialists (or extensively with medicine) : some can be disappointed by conventional medicine and its range of questionable products, some won't, … It's another dimension that should be studied more in detail when designing the risk communication.

The frequency of consumption and the budget allocated to them is also variable ; a lot of consumers are regular customers (37% of interviewees consume FS on a daily or weekly basis ; 30% "regularly" (at least one month every year) ; 35% "incidentally").

The monthly expense on FS is generally less than 40-50€ per month.

In order to better understand how FS consumption is qualified and defined by consumers, we propose to analyse those patterns of consumption with two models : the "medicine-intake model", and the "food-consumption model". Those are completely different in terms of practices of consumers, collective norms and representations, motivations, knowledge-building and networks of advice or "prescription" chains, etc.

Going on building the models, we could then break down the various patterns of consumption of "alternative health products" (FS or assimilated), that fall in those two categories, "medicine" and "food", along three dimensions :

(1) The relation with body :

- For medicine-view, it cures a sickness or an ache (that has to be previously felt by the ill person, through physical or physiological manifestations)
- For food-view, it nourishes a body that feels hunger, and that also has specific tastes and preferences, that adapts to activities (work, sport and leisure, …)

(2) The prescription :

- Strong and imperative for medicine, assessed by practitioners
- Weak for food, let at personal appreciation

(3) The relation with knowledge :

- Expert knowledge for medicine
- Common or unitiated knowledge for food (i.e. situated in natural categories or references such as family and personal history, traditions, etc.)

The notification process, as well as the known biological activity of some plant based food supplements, seem to implicitly treat FS consumption and related risk management along the "medicine" model, with the consequence (among others) of fearing risky auto-medication of consumers, their potentially challenging attitude against scientific recommendations, or their lack of will to listen to scientific advice. In other words, the underlying model or reference is ordered on expert knowledge, that has to define and teach "good practice", as opposed to a model based on preferences and taste, underlying food consumption.

On the other hand, we find the model (implicit as well) of food consumption, that we suppose is based on taste and "spontaneous appreciation" from consumers, of what fits them or what is a "healthy food".

For consumers, the vision of FS along either "medicine" or "food" doesn't seem as clear nor socially shared, even if the reference to "health" or physiological effects is strong in discussions (indeed, reference to health is also strong in the "food model"). Indeed, we can see that FS consumers, depending on their "profile" or "pattern of consumption", make original combinations of the three compounds detailed above. This is especially true for the profiles "well-being" and "performance".

PROFILES	Body	Prescription	Knowledge
Prevention	strengthening of defenses	medium	expert and „traditional"
Insufficiency / curative	treatment / supplement	relatively strong	expert
Well-being	harmony, long life, ...	weak (or coming from „alternative practitioners")	very large ; (pseudo-)expert, but also common sense
Performance	push the limits further	very weak	pseudo-expert

Figure 3 : Interpretation of the four profiles through the model of food and medicine qualification.

These "in between", hybrid combinations consequently seem to escape the management schemes, both of food and of medicine. The example of the consumer that is prescribed FS by his practitioner is at the opposite of the consumer who wants to improve his sport performances, after a sport-friend suggested him to do so. What is more complicated, is the example of a patient taking FS as medicine, that chooses to consume FS to cure a disease despite advice from his doctor, that may for instance follow advices of other patients that have the same symptoms.

We could consequently make two hypothesis :

– one on the heterogeneity of mode of consumption, or "consumption patterns", which justifies different policies adapted to them.
– the second on the emergence of a new type of consumption, that can't be qualified in a precise way, mixing compounds of the two models drawn. This is of course to be linked to the blurred, unshared definition and categorization of "health products" in general (may they be qualified as "alternative" or not). This would also justify the elaboration of specific policies.

A consequence of this is that it seems less important for risk management to distinguish between product types (or definitions), misunderstood by consumers, than between modes and patterns of consumption. (This will be discussed in section 3.2).

- **Low risk awareness from consumers, but who want to be better informed**

It can be noticed that most of the FS consumers seem very cautious regarding food and health, probably more than non consumers on the average. There is a sort of ambiguity in these attitudes, or a sort of unveiling of the various compromises consumers do, since they are at the same time interested in « natural », healthy and well-balanced diet, seem aware of risk concerns and « money » or lobby pressures from industry, but are nevertheless users of these products since they can alleviate problems they experience.

Risks associated with FS consumption are generally not spontaneously evoked by consumers, what lets us think that they are largely not aware that there simply *are* risks, but deplore what they perceive as weaknesses in the control. Some consumers seem to treat FS as "natural" products, that aren't seen very risky (between 30 and 50% of the respondents don't think there are risks associated with FS consumption, while the majority of other respondents think there are some (but don't necessarily know which risks, or how to act in face of them) ; see Figure 4).

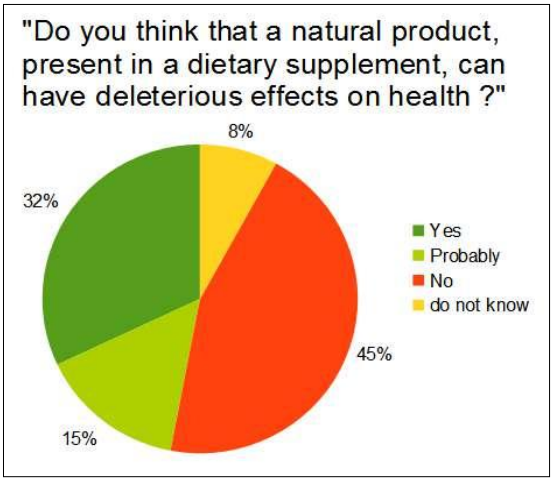


Figure 4 : Risk perception

FS are generally described positively, for example as "healthy" products or products that allow smartening up or improvement of one's performances ; they are sold over-the-counter, without prescription (can even be ordered on the Internet), allowing "self management" and allowing to save seeing the doctor ; etc. The main risk mentioned by consumers is overuse ("*excess is always bad*"). This observation underlines that a communication on risks associated with FS should be "positive" rather than "negative" (only pointing out risks, disqualifying consumers attitudes and knowledge), in order to avoid a gap between communicators and "targets", the last seeing FS with such a "positive aura".

On the other hand, a large part of interviewees don't seem to be aware that simultaneous intake of drug or food could pose a health risk (around one out of four think that FS are always compatible with drug intake ; see Figure 5). We can though say that there is a kind of underestimation of danger concerns among our sample, for "inner" risks (environmental contaminants, ...), as well as for interaction risks.

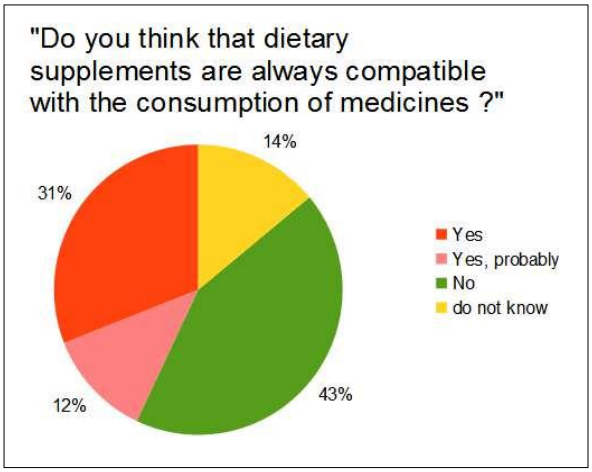


Figure 5 : Perception of interaction risks

About FS, people can trust relatives or sports friends as much as general practitioners. The latter are criticized to generally show a lack of knowledge, will and time to consider seriously FS consumption (considering all the available products and moreover potential interactions). It was remarkable to notice that every of the consumers that attended the focus groups have had problems to talk of their FS consumption with their practitioner, and raised the difficulty to find a competent specialist that is at the same time open to those "alternative health products".

During focus groups, consumers deplored the lack of knowledge on the long-term side-effects of FS, revealing lacks in risk management and call for expert assessment.

Though there doesn't really appear to be a strong demand for more control or direct protection (excepted from more "active" consumers), the demand for trusted expert assessment and information on risk is quite strong and seems better accepted than formulation of "good practice" or bans. This demand for more information regards concentrations in active compounds, precise composition, quality tests passed (certification, …), notice of use, origin of raw materials and place of manufacture, … This demand for more information is supported by the results, which show that a majority of consumers read the available information (leaflets, when there is one)(see Figure 6).

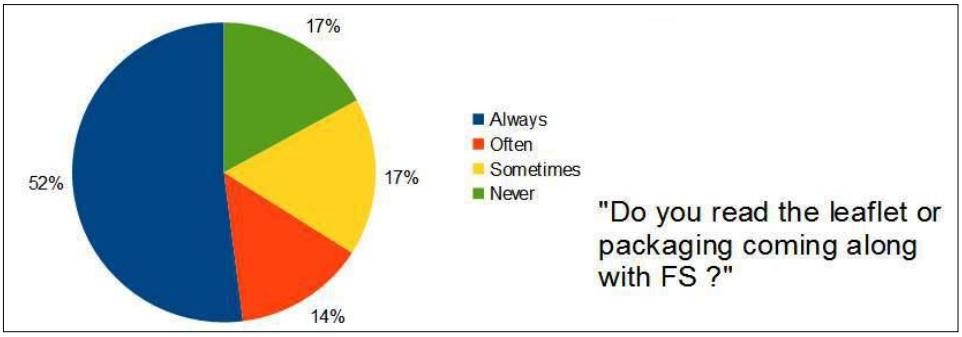


Figure 6 : reading of labels or leaflets provided with FS

Concerning the Foodinter research project, they felt rather dubious about the expected results of laboratory research and asked for a good communication of these results to the public.

From all these results we can conclude on a hypothetical way that, even if FS consumption is growing, consumers do not entirely trust commercial food nor medicine. FS are rather clearly distinguished from drugs and from food, even if consumers don't seem to know clearly how to treat them (as medicine, as « complements » or « supplements », as convenient « boosters », ...). 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.

- **Which status to give to consumers in face of risk ?**

What should be discussed by the public authorities is the status of consumers in face of risks. Are they only "passive receptors", to inform or "educate" through expert advice ? (like in Brown's deficit model (Brown, 2009)). This model and presuppositions, such as the idea of direct change from the "targets" (even if the message comes from a trusted and independent source), appears to be invalidated through our discussion groups : *"even if there are risks, I don't know if I will stop directly, I can still choose..."*

...Or shall we take their practices, representations and reflexivity (opinion, recommendations, …) into account, in a risk governance regime that is more symmetric (as opposed to the unilateralism of Brown's "deficit" and the "new deficit" models) ?

Consumers don't just have perceptions and passive reactions ; they make reorganizations and arrangements.

Consequently, what about thinking on this "adaptive risk governance regime", that Brown pointed out ? This should be built on the features of risks of "Second Modernity" rather than being overtaken by them ?

This of course doesn't exclude the need for expert assessment and recommendations, but the limits of this assessment should be made explicit and communicated, while the modalities of interactions between consumers and experts should be redefined in order to rebuild trust and avoid gaps between them.

Moreover, we think that such an "ideal" communication process (which, to caricature, supposes exhaustive and uncontroversial assessment, ideal and clear message, ideal comprehension and application from the "public", leading directly to behaviour change) could hardly find grip on consumers as long as they are seen as "mean consumers", or "consumers to educate", "whose practices are wrong and to be changed"... And thus risks to remain a pious hope. Moreover, this struggle is supposed to occur every time a new risk concern will appear, what is clearly unpractical and increases each time the risk of defiance !

Consumers are not "mean", nor "passive", nor stupid ! They mostly have a reflexivity on their FS consumption, which can sometimes take them a lot of their daily time. They want to "master their consumption", and can not blindly trust any one (who ever it can be, even the practitioner himself !)...excepted maybe their own body and feelings. They can have an active attitude, or a critical attitude against a communication that would target "mean" consumers in which they would not recognize themselves, or inversely would feel stereotyped. We can also formulate

the hypothesis that FS consumers have a "culture of health" superior to the average, as they can be more reflexive and critical, are active and accumulate knowledge, are getting used to listen to their bodies and draw conclusions from their consumptions, … If this happened to be proven, it would imply that a simplistic or paternalist communication strategy would be quickly dismissed by those consumers !

All this call for taking consumers' specificity into account is globally positive for science, as consumers don't show a global mistrust in science (this is indeed the opposite, as scientists were given a lot of credit during our focus groups), but the main question behind this trust is to know in whose name they speak ! This raises the important need for more independent, quality assessment and advice.

- **Which type of risk management could fit the complexity of FS risks issues ?**

Presently, risks associated with FS are managed by a system of "auto-control", inherited from quality and standards systems of "past" era. We qualify this era as "past" in reference to Ulrich Beck (Beck, 1986 ; 1991) since the nature of risks feared by consumers (in particular, long-term effects and interaction risks) and studied in the Foodinter research largely overtake the usual framing of risks applied from decades on, which focused on precise, bounded and "simple" risks. These risks were "simple" since it was easy to identify their causes and their effects or consequences, objectified through scientific assessment, and allowing to assign responsibilities to actors without doubt.

In contrast, the "new era" we entered into is characterised by uncertainty. It bares a renewed nature and way of qualification of the risks having to be managed, such as the interaction risks studied in Foodinter (but the same is true for long-term effects of FS consumption, not studied here) : risks are complex or multi-factorial, "systemic" and retroactive as for interactions, fast-evolving and depending on a huge range of products or substances, …. It has become nearly impossible to clearly determine and isolate causes or factors of risks, while the same is true for consequences which depend on a potential infinity of particular cases (here, of consumers, practices, nutritional regimes, ...). In this context, it appears clearly that the regime of responsibility can't be that simple than in the "past". Responsibilities are shared, impossible to isolate.

These considerations are also perceptible from the discussion groups conducted with consumers, which showed they were far from being all dupe of the inherent limits of the actual risk management model.They can even become more aware of these limits when scientists, public actors or enterprises try to present only certitudes to them... and make silence on everything that remains unclear or uncertain.

Moreover, we can add to these the following facts : first is that FS are sold over-the-counter, and that this status seems hardly modifiable ; and secondly, that

there is absolutely no control of the Internet (advice and sale), and it is clearly impractical to prevent consumers from buying FS on this platform that offers them numerous advantages.

What consequently seems to become unavoidable is that we have to reconsider the ideal model of "total control" of risks, which clearly shows its limits in the era of "Second Modernity" (Beck, 1986 ; 1991) and in face of corresponding characteristics of risks. We can't however say that we have to definitely turn away from it (it's not our responsibility, and would mean that everything can be thrown away in it, which is not the case ; control would still be an important dimension of an innovative, adaptive and multi-dimensional risk management system).

The question of trust from consumers is a central point to consider when we make the assumption for the need of an adaptive, symmetrical risk governance regime, as it seems that critics directed to the actual risk management system can only go growing with future occurrences of unanticipated risks. To rebuild this trust, we propose a risk management approach that makes its limits explicit, that implies citizens and consumers through dialogue, and that is fundamentally more anticipative and adaptive (especially of what's yet uncertain and even unknown !).

The place of each category of actor concerned by risks associated with FS (consumers, but also health professionals, producers, and scientists as well) could be redefined in face of the new nature of risks having to be managed.

The communication associated with this system shouldn't aim one "mean" target, but specific profiles, and should also be done at various levels to reach the expectancies of very different consumers, it should be multiple. It should be complete, deep and simple at the same time, should allow consumer empowerment, by allowing them to put risk in perspective and to increase their reflexivity (on risks as well as on risk management system), but also give them simple, conventional practical tips or examples.

2.2 Collection of samples, labelling and notification status

The selection, for further studies, of products sold on the Belgian market as « food supplements » was carried out by taking into consideration the following criteria:

1) Active ingredients potentially susceptible to interact with some key enzymatic systems such as those involved in phase 1 en 2 metabolism. According to 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 and traders, mentioned by consumers during the surveys or mentioned in many commercial advertisements)

3) Products more prone to be contaminated by several kinds of contaminants (plant toxins, mycotoxins, heavy metals, PAH, dioxins and PCB, pesticides). Following this criterion, plant based products obtained by 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 to the information gathered, 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 products all made from one specific plant material and complying with criterion 1. In addition, four of them were in accordance with criterion 2.

The final selection was made of six different categories of 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
- **Saint-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):** Said to increase libido and limits sexual disorders; plant toxins (alkaloids); less studied
- **Black radish (R):** Stimulation of bile secretion and of intestinal activity; plant toxins (glucosinolates); less studied

During the first trimester 2007, 61 samples were purchased via internet (36 samples), pharmacies (18 samples) and specialized shops (7 samples). Eighteen were notified in Belgium while 43 were not. Forty-two were obtained as capsules containing solid plant material or extract, 13 were tablets and 6 were available as oily capsules.

The complete list of the 61 samples is presented in the final report of phase I of this project (Mormont et al, 2009).

We know that this selection of products is not statistically representative of the Belgian market of food supplements, but it allowed us to take a picture of what the Belgian consumer was able to find on the market in 2007. It is important to note that the conclusions drawned from the study of these 61 samples can therefore not be extrapolated as such to the whole field of botanical food supplements, at the present time (2011).

Notifications status

Taking into consideration the available information on the notification, the products under study have been classified into 3 groups, in agreement with Pascale Degrijse, from the Federal Public Service Health, Food chain safety and Environment, Animal, Plant and food directorate-general, DG:

1. The actual food supplements (FS), which are notified products that comply legislation,
2. The not notified products (NNP), for which no notification file has been accepted by the Belgian authorities, and thus cannot be named food supplements,
3. The medicinal products (MP), which are notified products that cannot *stricto sensu* be named food supplements because of one or a combination of the following reasons:
   - medicinal claims: the product is presented as having curative or preventive properties for human diseases,
   - the product exceeds the maximum permitted contents of active ingredients,

The MP, as well as the NNP, are not legal on the market, unless they were registered as drug. None of the products bought in the framework of FOODINTER was registered as drug.

Two preliminary results of our shopping are interesting:

- ✓ Out of 25 products bought in pharmacies and specialized shops, 11 were not authorized to be on the Belgian market at that moment (3 NNP, 8 MP). This means that some products pass the barriers and reach the market's stands. The argument of the long period required to have the notification is sometimes evoked. The FASFC performed the same kind of sampling at the end of 2007; they found approximately 30% of not notified products, consistent with our observation.
- ✓ On the other hand, only 4 of the products bought through the internet were notified (out of 36), and thus may not be sold in Belgium.

Labelling

From the 61 chosen products, the presence/absence of several indications on the label was analyzed:

- Is the denomination "food supplement" indicated?
- Are the name and scientific name of the plant provided?
- Is the advised consumption (frequency) indicated?
- Is the net weight provided?
- Are the active ingredients concentrations indicated?
- Is the list of ingredients present?
- Are nutrition facts mentioned ? **
- Is there any expiration date?

- Are there recommendations for a good conservation?
- Is there information about keeping out of reach of children?
- Is the NUT/ Lot number/ charge number present?
- Is the name and address of the furnisher/seller present?
- Is it mentioned that the food supplement cannot replace a normal diet?
- Is there a phone number / e-mail / website provided for more information ? **
- Does the packaging provide information about the necessity to not exceed the advised dose, to consult a doctor *, to be aware of the drug interactions*, or to warn the persons who are more subject to health issues *?

* Not requested but seems important for us in the specific frame of this project
** Not requested, but might be interesting information

Some questions arose from the large variability of the information on the package: Is a postal box a sufficient address? Is the notation "complement" similar to "food supplement"? Is "60*500mg" sufficient as a net weight?

The situation about the labelling of products sold as food supplements on the Belgian market can be considered as reassuring information for the notified food supplements. Nevertheless, two of those FS have insufficient labelling, lacking 3 mandatory information. It has to be stressed that indications on active ingredients concentration is difficult to judge since the active ingredients are sometimes even not known. As an example, none of the Maca plant extracts provides any concentration of potentially active ingredients.

For some FS, NNP and MP, the advice to consult or to speak to the doctor is not mentioned on the label. We stress in this project that this is a matter of concern, since interactions with drugs are widely known by the professionals of the medical sector. Those interactions will be approached in the following chapters. Nevertheless in the Belgian legislation (RD 27/08/1997), safety warnings are mandatory for certain foods and food supplements containing plant preparations.

- the labeling of food supplements containing the leaf of *Ginkgo biloba* L. must include the following warning: "Consult your doctor if you take anticoagulant medication."
- the labeling of food supplements containing the flowering aerial parts of Hypericum perforatum L. must include the following warning: "Inform your doctor and/or pharmacist if you are taking medicine simultaneously.".

This Belgian legislation also limits the presence of certain plant constituents in FS.
For example :

- Food supplements to which the seed or the seedling of Glycine max. (L.) Merrill have been added, may only be marketed under the following condition: the intake of the daily dose as recommended in the labelling or in advertising may not result in an intake quantity of isoflavones exceeding 40 mg (expressed as glycoside of the main component).

- Food supplements to which the leaf of *Ginkgo biloba* L. has been added may only be marketed under the following condition: the intake of the daily dose as recommended in the labelling or in advertising may not result in an intake quantity of flavonol glycosides exceeding 21.6 mg and an intake quantity of terpene lactones exceeding 5.4 mg.
- Food supplements to which flowering aerial parts of Hypericum perforatum L. have been added may only be marketed under the following condition: the intake of the daily dose as recommended in the labelling or in advertising may not result in an intake quantity of hypericine exceeding 700 µg.
- Maca can be in food supplements if analysis reports prove that the plants in question are free of alkaloids

2.3 Analysis of environmental contaminants in selected food supplements

2.3.1 Trace elements

Metals and minerals are integral parts of the food chain, and, even if they have different origins (natural, artificial), they reach the lasts steps of such chains, thus becoming in several cases very dangerous with irreversible effects for animal and human life (Locatelli, 2008). The toxicity depends on the doses ingested by the human being. For heavy metals, chronic diseases occur when the consumer is exposed during a long period. Those metals are mainly found in our food, in plants, meat, fish and water. The risks have decreased thanks to the environmental actions taken by the authorities during the last decades (unleaded gas, collection of worn batteries, unleaded paints,…).

Nevertheless, even if our food can be considered as safe, any other intake should be avoided as far as possible.

2.3.1.1 Analysis of trace 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). The detailed methodology and results are available in the final report of phase I of FOODINTER (Mormont et al., 2009).

The results reveal that there were 10 non compliant (NC) samples with respect to the Belgian legislation for toxic elements in FS, in force in 2007 (7 NC samples for Pb and 4 NC for Cd; one sample exceeded the maximal limit for both elements). See the results in TABLE I.

TABLE I: Heavy metal concentration (µg/kg) in non compliant samples, according to the Belgian maximal limit in force in 2007. FS : Food supplements (notified products), MP: medicinal products, NNP: not notified products. - : not detected.

			Hg	*As*	*Cd*	*Pb*
	Belgian maximal limit (µg/kg)		**200**	**1000**	**500**	**1000**
Brand name	***Product name***	***Type***				
Mattisson Healthcare	Active maca	FS	-	-	**931**	-
Bio-Life SPRL	GINGKO BILOBA 400	MP	-	-	-	**6707**
Laboratoires Feniouxl SPRL	MILLEPERTUIS FORT	FS	-	-	-	**13020**
Laboratoires Feniouxl SPRL	Maca	FS	-	-	**552**	-
Dieti Natura	Ail	NNP	-	-	-	**2314**
Dieti Natura	Maca	NNP	-	-	**996**	-
Dieti Natura	Millepertuis	NNP	-	-	**958**	**73870**
Dieti Natura	Ginkgo biloba	NNP	-	-	-	**3202**
Maximum Nutrients	St John's Wort 60	NNP	-	-	-	**2107**
Vitadyne	Millepertuis	MP	-	-	-	**1412**

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

In July 2008, maximal limits for heavy metals in food supplements have been included in the European Regulation (Regulation (EC) n° 1881/2006), and are the following :

Hg : 100 µg/kg

Pb : 3 000 µg/kg

Cd : 1 000 µg/kg (except FS derived from seaweed and from dried bivalve molluscs : 3 000 µg/kg)

According to this new legislation, only 4 products would remain non compliant out of 10.

2.3.1.2 Cadmium in FS: health outcomes

- Because of its high rate of soil to plant transfer, cadmium is a contaminant found in most human foodstuff, which makes diet the primary source of exposure among non smoking, nonoccupationally exposed populations (Clemens, 2006). In the human body, cadmium has no known physiological function, and no mechanism is expected to have evolved for its selective transport and homeostasis. Most of the cadmium is absorbed through intestinal transit. Following the literature, from 3 to 7% of the cadmium present in foodstuff is absorbed (Satarug et al.,2010). The food groups that contributed to the major part of the dietary cadmium exposure, primarily because of the high consumption, were cereals and cereal products, vegetables, nuts and pulses, starchy roots or potatoes, and meat and meat products (EFSA, 2009). In Belgium, Vromman and co-workers showed that Cereal products and

potatoes are the main food groups that contribute to Cd exposure in Belgium, while offal (which are not regularly consumed) only contributed to 0.57 % of the exposure and crustaceans and bivalves to 4.01 % (Vromman et al., 2010).

The other way to absorb cadmium is the inhalation of industrial smokes, cigarette smoke, and urban air. Cadmium exposure is well studied and documented, considering both high and normal exposure levels. In their review, Satarug and co-workers cited different consequences of exposure to cadmium such as osteoporosis, renal injuries, diabetes, hypertension, cancer, etc. (Satarug et al., 2010) (FASFC, 2009a).

The European Food Safety Authorities (EFSA) defined in 2009 a new safe provisional tolerable weekly intake (PTWI) of 2,5 µg cadmium/week/kg body weight. This health based guidance value has been discussed several times in the past, arguing that the past safe intake level does not provide sufficient health protection and that it should be lowered.

The results observed for the products analyzed in this study were converted to µg cadmium per week per kg body weight, considering a body weight of 70kg (see TABLE II). The maximum intake following the recommendations provided on the label is far from being negligible (up to 14% of the maximal intake).

TABLE II: Cadmium intake from the ingestion of non compliant samples for Cadmium. FS: Food supplements (notified products), NNP: not notified products, Cd=Cadmium, PTWI=provisional tolerable weekly intake.

Brand name	*Product name*	*Type*	*Cd/caps*	*recommended consumption*	*Cd/day worst case*	*Cd/week*kg bw* **PTWI: 2,5**	*% PTWI*
			µg	*# caps*	*µg/day*	*µg/week/kg bw*	
Mattisson Healthcare	Active maca	FS	0,72	2	1,43	0,14	6%
Laboratoires Feniouxl SPRL	Maca	FS	0,18	6	1,09	0,11	4%
Dieti Natura	Maca	NNP	0,40	9	3,58	0,36	14%
Dieti Natura	Millepertuis	NNP	0,25	6	1,49	0,15	6%

Even if the cadmium levels measured in the products of this study are too low to cause injuries, they should be added to the amount taken up with the normal diet, which has been calculated to be 1,27 µg/kg bw/week (FASFC, 2009a). Moreover, because of the high variability in the conditions of plant cultivation and the lack of systematic control at the production site, high doses of cadmium could occur.

2.3.1.3 Lead in FS: health outcomes

Lead contamination of the human body results from various sources such as lead dust, lead-containing objects such as paints and lead pipes, through the gastrointestinal tract from contaminated water or vegetal food contaminated after atmospheric deposition (some plants are capable of taking up lead from soil through their root systems, but this uptake does not appear to be appreciable (EFSA, 2010),

and from several industrial processes such as glazing or manufacture of lead accumulators (Rosin, 2009).

In the body, 95–99% of ingested lead is sequestered in erythrocytes and dispersed through soft tissues and bone and is also found in hair and nails. The metabolism of lead stored in bone parallels that of calcium and the resorption of calcium during osteoporosis is accompanied by passage of lead from bone into blood. In blood, its half-life is up to 30 days and it is excreted in the urine; its storage in bone can remain for more than 20 years (Rosin, 2009).

Based on consumption data from the Belgian food survey from 2004 and results from the Pb analyses control in the framework of the control program from the FASFC for 2006, 2007 and 2008, the median and the 95th percentile dietary exposure to lead in the Belgian adult population was estimated at 0,9 µg/kg bw/week and 2,5 µg/kg bw/week, respectively. The median and the 95th percentile dietary exposure of children was estimated at 2,9 µg/kg bw/week and 7,5 µg/kg bw/week. The Pb dietary exposure for consumers that follow the food recommendations for the consumption of vegetables and fish was estimated at 0,98 µg/kg bw/week and 1,02 µg/kg bw/week, respectively (FAFSC, 2009b).

Considering the multiple diseases correlated with lead ingestion, the World Health Organization (WHO) defined a safe provisional tolerable weekly intake (PTWI) of 25 µg lead/week/kg body weight (WHO, 2000). More recently, the EFSA (EFSA, 2010), as well as the JECFA (2011) considered the PTWI as no longer appropriate, because, based on the dose–response analyses, the JECFA estimated that the previously established PTWI of 25 µg/kg bw was associated with a decrease of at least 3 IQ points in children and an increase in systolic blood pressure of approximately 3 mmHg (0.4 kPa) in adults. The approach is to use the Benchmark Dose Lower Confidence Limit (BMDL) calculated on the basis of the modelization of a dose/response curve in animal experiments, or from human epidemiology studies (which was the case in the EFSA and JECFA avices on heavy metals), as suggested by the International Programme on Chemical Safety (ICPS, 2009a,b). The BMDL varies according to the endpoint that has been considered in toxicological studies. To evaluate the risk, a margin of exposure (MOE) is calculated, which is the ratio between the BMDL and the intake. The EFSA concluded that the risk of clinically important effects on either the cardiovascular system or kidneys of adult consumers is low to negligible at current levels of lead exposure. In infants, children and pregnant women, there is potential concern at current levels of exposure to lead for effects on neurodevelopment. Protection of children and women of child-bearing age against the potential risk of neurodevelopmental effects should be protective for all other adverse effects of lead, in all populations (EFSA, 2010).

The seven non compliant products according to the Belgian regulation in force in 2007 are presented in Table III . Except for one garlic sample, lead was detected

only in samples prepared from the leaves, fruits or barks of the plants cultivated outside (St John's wort and *Ginkgo biloba*), consistent with the fact that lead in plants is due mainly to atmospheric deposition or absorption by their external parts (Albertine, Oetterer, & Prado Filho, 1997).

The weekly intake has been calculated following the recommended consumption on the labelling (the worst case has been chosen).

Let's note that the Belgian adult median dietary exposure to lead via normal food has been estimated to 0.9 µg/kg bw/week (Vromman et al, 2010).

TABLE III: Lead intake from the ingestion of non compliant (NC) samples for Lead (according to the Belgian legislation in force in 2007). FS: Food supplements (notified products), MP: medicinal products, NNP: not notified products, Pb=Lead, PTWI=provisional tolerable weekly intake; BMDL=Benchmark dose lower confidence limit (4,4 µg/week*kg bw); MOE = Margin of Exposure (worst value for adults has been chosen). PTWI = 25 µg /week*kg bw.

Brand name	Product name	Type	Pb/caps µg	Recommended consumption # caps	Pb/day worst case µg/day	Pb/week µg/week*kg bw	% PTWI	MOE
Bio-Life SPRL	Ginkgo Biloba 400	MP	2,68	2	5,37	0,54	2,1%	8,2
Laboratoires Feniouxl SPRL	Millepertuis Fort	FS	2,47	6	14,84	**1,48**	**5,9%**	**3,0**
Dieti Natura	Ail	NNP	0,71	6	4,23	0,42	1,7%	10,4
Dieti Natura	Millepertuis	NNP	19,21	6	115,24	**11,52**	**46,1%**	**0,4**
Dieti Natura	Ginkgo Biloba	NNP	0,91	6	5,48	0,55	2,2%	8,0
Maximum Nutrients	St John's wort 60	NNP	0,99	3	2,97	0,30	1,2%	14,8
Vitadyne	Millepertuis	MP	0,62	3	1,86	0,19	0,7%	23,6

The St John's wort DIETI NATURA exceeds 2,5 folds the BMDL, giving a margin of exposure (MOE) of 0,4, which is too low, according the safe MOE proposed by EFSA, of a minimum of 1 (EFSA, 2010), and contributing to nearly half of the PTWI. The other products seem to contribute less severely to the weekly intake.

Considering the contribution of other lead sources (air, water and food), which for an adult is estimated from 0,9 µg/kg bw/week to 2,5 µg/kg bw/week (thus from 4 to 10 % of the PTWI), all the products mentioned in Table III do cause a significant contribution to the lead exposure.

The intake considered here is far from the historically excessive exposures to lead among the upper class of the Roman society, or in the 19th century with the extensive use of lead in many industrial processes in Britain, nevertheless the long term consequences of exposure to lead are still major: degenerative brain disease, degenerative vascular disease, hypertension and vascular disease (Rosin, 2009).

Considering those results, the lead and cadmium content in products presented as food supplements for the consumer could be a matter of concern.

These results show that it is important to not forget the food supplements consumption in lead and cadmium risk assessments studies.

2.3.2 Mycotoxins

A mycotoxin is a toxic secondary metabolite produced by molds. The reason for the production of mycotoxins is not completely understood. Because mycotoxins weaken the receiving host, the mold may use them as a strategy to better prepare the environment for further fungal proliferation. The production of toxins depends on the surrounding intrinsic and extrinsic environments. The presence of mycotoxins in food (mostly cereals and plant based products) is due to multicausal factors including environmental conditions (climate and insects as fungal spore vectors for example), improper agricultural practices, wrong post-harvest handling, no specific HACCP (Hazard Analysis of Critical Control Points) and scarce implementation of decontamination procedures (Brera et al. 2008). Temperature treatments, such as cooking and freezing, do not destroy mycotoxins (Cahagnier, 1998).

Around a thousand mycotoxins have been described, but only some families are of concern for humans and animals (e.g. aflatoxins, fumonisins, trichothecenes, ochratoxins). They may have various effects as for example carcinogenic, provoking acute hepatitis, poisoning and renal pathologies (Cahagnier, 1998).

Mycotoxins: possible health concerns

The occurrence of mycotoxins in the products under study was investigated 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 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. A detailed description of the analytical method can be found in Diana Di Mavungu *et al*. (2009).

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 products analyzed, none of the 23 mycotoxins was detected. The toxins FB1, FB2, FB3 and OTA were detected in some samples (Table IV).

Nevertheless, the levels of FB1, FB2 and FB3 were largely below 800 µg/kg (EC maximal limit for the sum of FB1 and FB2 in breakfast cereals, Regulation (EC) n° 1881/2006) in all samples. In 2 samples, OTA was found at a level above 2 µg/kg (EC maximal limit for wine and grape juice, Regulation (EC) n°1881/2006).

TABLE IV: Mycotoxin contamination (µg/kg) in samples. FS: Food supplements (notified products), MP: medicinal products, NNP: not notified products

Brand name	***Product name***	***Type***	*FB1*	*FB2*	*FB3*	*OTA*
Mannavita BVBA	Kyolic	MP	<1	<0,3	<1	**6**
Biodynamics BVBA	MACA 500	FS	<1	<0,3	<1	**2,5**
Pierre Fabre santé Benelux SPRL	Elusan Ail	FS	10	8	3	1
Orthonat (Vitanutrinat)	Isoflavones 50	MP	4	<1	<1	<0,3
Dieti Natura	Radis noir	NNP	4	2	<3	<0,3
Netlab pharma	Meno (jour)	NNP	<1	<0,3	<1	1
Netlab pharma	Meno (nuit)	NNP	<1	<0,3	<1	1

The fact that the maximal limit set by the European authorities is not exceeded in the foodstuffs may not be considered as a sufficient guarantee for food safety, because there are still a lot of uncertainties linked to the toxicological assessment of the contaminants. Among these, it is frequently argued that the possible additive and, worse, synergistic effects of different contaminants are not taken into consideration. This is the case here as at plausible intestinal concentrations, some polyphenols and dietary contaminants, such as captan (pesticide) or cadmium (heavy metal), markedly increase the mycotoxin ochratoxin A absorption (Larondelle et al., 2006). Nevertheless, interactions with the bolus in the digestive system (stomach, intestine) would probably change the mycotoxin concentration in contact with the intestinal cells.

Recently, the EFSA evaluated the daily intake of OTA from food, in Europe. This mean intake was evaluated to 2-3 ng/kg b.w./day for adults (15 years old and more) and 6-8 ng/kg b.w./day for high consumers (EFSA, 2006). In the same study, the EFSA proposed a maximal tolerable intake set to 120 ng/kg b.w./week. At present the weekly exposure ranges from 15 to 60 ng OTA per kg bodyweight per week, including high consumers of foods containing OTA, which is below the TWI value of 120 ng/kg b.w. Nevertheless, more data would be needed to assess exposure rates of infants and children.

In opposition with what is observed for the heavy metals cadmium and lead, the products presented as food supplements for the consumer considered in this study do not significantly contribute to human exposure to individual mycotoxins.

2.3.3 Polycyclic Aromatic Hydrocarbons (PAHs)

2.3.3.1 Definition and health outcomes 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. PAHs are formed during the incomplete burning of organic substances and pyrolysis processes (Mc Grath et al., 2001) such as: forest fires, processing of coal and crude oil, vehicle traffic, residential heating, industrial power generating, cooking, smoking (Tamakawa, 2008). They are widely present in the environment due to their lipophilic properties allowing their adsorption on atmospheric particles and their deposition in sediments, soils and plants. The primary routes for human exposure to PAHs are inhalation of polluted air, foods normally containing microgram quantities of PAHs (smoked foods for example), and foods whose contamination is due to the environment and/or production practices, such as a drying step before grinding of a plant. Among several routes, diet is estimated to be the major pathway for human exposure (Tamakawa, 2008).

In the European Union, 16 PAHs have been selected as priority substances for food monitoring. These PAHs were selected on the basis of their classification as having 'probable' or 'possible' carcinogenicity to humans (European Commission, 2005).

Products presented as food supplements for the consumer may contain large contaminations with PAHs. The risk associated with PAHs in those kinds of products was recognized by the Scientific Committee on Food (SCF, 2002) but further investigation is needed to fix maximum tolerable levels. The critical effect of PAHs is their carcinogenicity. Because some of them are genotoxic, it is impossible to determine threshold concentration. There is currently no legislation in place controlling the maximum levels of B[a]P or other PAHs in food supplements. In the meantime, maximum tolerable levels are defined for benzo[a]pyrene (B[a]P) and benzo[a]anthracene (B[a]A) in smoke condensates (Regulations (EC) N° 2065/2003) and for B[a]P in various foodstuff categories (Regulation (EC) N° 1881/2006). Presently, B[a]P is used as a marker for the occurrence of carcinogenic PAHs. For example, the maximum levels for B[a]P in oils and fats are fixed to 2 µg kg^{-1}. Maximal limits are also set for smoked fish and meat, as well as for babyfood and bivalve molluscs, for which the maximal limit is the highest : 10 µg kg^{-1}.

The main problem in evaluating carcinogenicity of PAHs in food and environment is that they are not present as individual compounds but as complex mixtures. This could be solved by summing PAH concentrations and expressing them as B[a]P equivalents after application of a toxic equivalent factor, similar to the procedure for dioxins. However, this is not possible for PAHS, because not all PAHs are acting through the same pathway.

In a recent advice, the EFSA (EFSA, 2008) came to the conclusion that BaP is not a suitable indicator for the occurrence of PAHs in food. Based on the currently available data relating to occurrence and toxicity, the CONTAM Panel concluded that PAH4 (B[a]P + benz[a]antracene + benzo[b]fluoranthene +chrysene) and PAH8 are the most suitable indicators of PAHs in food, with PAH8 (B[a]P + benz[a]antracene + benzo[b]fluoranthene + benzo[k]fluoranthene + benzo[ghi]perylene + chrysene + dibenz[a, h]antracene + indeno[1,2,3-cd]pyrene) not providing much added value compared to PAH4.

Based on this EFSA advice, the European Commission has drafted an amendment to regulation (EC) No. 1881/2006 with revised limits for benzo-a-pyrene and certain food stuffs and with additional limits for the PAH4 (European Commission, 2010). However no limits for food supplements will be included in the next change of regulation (EC) n°1881/2006.

2.3.3.2 Analysis of PAHs

During phase 1 of this project, 16 PAHs were quantified in the 61 products bought, using high performance liquid chromatography coupled to an ultraviolet, diode array or fluorescent detector as described in Danyi, et al. (2009). Detailed results are available in the final report of the phase I of the present project (Mormont et al, 2009).

The results showed that S^{t}-John's wort and *Ginkgo biloba* extracts presented the most frequent contaminations and the highest average values for PAHs concentrations. Generally, not notified products bought through internet showed higher contaminations.

In order to perform a risk assessment, in phase 1 of this project (Mormont et al., 2009), we calculated the daily intake of the sum of the 16 PAHs from the products taking into account their PAH concentrations and the recommended ingestion per day of each analyzed product, and compared it to the current margin of exposure (MOE) used by the JECFA (2005). The MOE has been calculated by dividing the PAH BMDL level by the PAH intake from each product. According to EFSA (2005), an MOE of 10,000 or higher, if it is based on the BMDL10 from an animal study, would be **of low concern** from a public health point of view and might be considered as a low priority for risk management actions. Two of them were measured with a MOE lower than the safety limit of 10 000. In our study, the PAHs intake from two products would lead to an MOE below 10 000.

As an exercice, the MOE has been calculated based on the daily mean intake of B[a]P and PAH8 (one of the most suitable indicators according to EFSA (2008)) in a normal diet plus the intake from each product, and taking into account the specific BMDLs for B[a]P and PAH8. The respective daily intakes from a normal diet are estimated at 315 ng/day for B[a]P, and 2405 ng/day for the PAH8.

For each of the 61 products, the calculated MOE was higher than 10.000 (Results not shown).

However, it is interesting to note that the daily intake of B[a]P and/or HAP8 from the products varies from 0 to 35% of the average intake from a normal diet. The majority of samples (55 out of 61) contribute for less than 5% to the daily intake of B[a]P and/or HAP8, while 1 and 2 samples only contribute to more than 20% of the B[a]P and HAP8 daily intake respectively (see Figure 7).

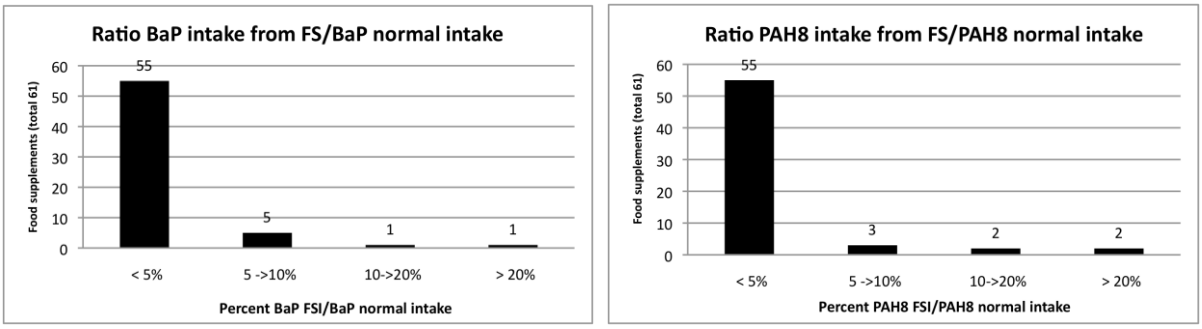


Figure 7 : Number of samples per categories of percentage of the daily intake of B[a]P and PAH8. "Percent BaP (PAH8) FSI/BaP (PAH8) normal intake" : ratio between the BaP (PAH8) daily intake from FS and the BaP (PAH8) daily intake from normal diet.

We see in Figure 7 that the probability to reach more than 5 % of the average intake of a normal diet is not negligible for both BaP and PAH8, which makes the PAH content a possible matter of concern.

2.3.4 Organochlorine pesticides (OCPs), polychlorobiphenyls (PCB's), polybromodiphenylethers (PBDEs) and dioxins

2.3.4.1 Definition and health outcomes

Persistent organic pollutants (POPs) are chemical substances that have long half-lives in air, soil, sediments or biota and thus persist in the environment. Because of their high lipophilicity, they can contaminate all food chain levels. They are toxic and cause adverse health effects to humans and wild life. This group includes in particular OCPs (organochlorine pesticides), PCBs, (polychlorobiphenyls) PBDEs (polybromodiphenylethers) and dioxin. They have different origins: released as agrochemicals and industrial substances, or accidentally as by-products from combustion processes (Scippo et al., 2008). Except for occasional occupational exposures, the main intake road of POPs for humans remains the food. Foodstuffs of animal origin account for more than 90% of the human exposure.

Numerous adverse health effects are attributed to POPs. Several organochlorine pesticides are suspected to act as endocrine-disrupting compounds (EDC). Like those measured in this study, they might contribute to numerous human female reproductive disorders and emphasize the sensitivity of early life-stage

exposures (Crain et al., 2008). Crain & co-workers suggested that interference with hormonal regulation of the menstrual cycle resulting in long and irregular cycles will reduce fecundability (the ability to conceive in a menstrual cycle). Several POPs and PCBs are also suspected to be human carcinogens. For example, it is well known that dioxins are carcinogenic in several animal species and humans (Scippo et al., 2008).

2.3.4.2 Analysis of organochlorine pesticides (OCPs), polychlorinated biphenyls (PCB's), polybromodiphenylethers (PBDEs) and dioxin

For the analysis of OCPs, PCBs and PBDEs in food samples, only the six oily samples were selected for analysis: four Garlics, one *Ginkgo biloba*, and one St John's wort. 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), using gas chromatography coupled to mass spectrometry (GC-MS).

Methodology for detection and detailed results of the analysis of OCPs, PCBs and PBDEs are described in details in the final report of phase I of the present project (Mormont et al, 2009).

Dioxins were measured in the same oily samples using the CALUX method, according to Focant et al. (2005).

None of the OCPs and PBDEs was detected above the limit of quantification (LOQ). Concerning the dioxins, all the samples were below the LOQ. This appears like reassuring results, and is consistent with the fact that POPs are known to bioaccumulate mostly in fatty tissues of animals and to a lesser extent, in plants.

2.3.5 Food contaminants and secondary plant components: interactions at the intestinal level

The intestinal mucosa cell monolayer is also the first barrier that either allows or prevents the entry of food antigens including food proteins, commensal gut microorganisms and pathogens, into the underlying tissues. It becomes increasingly evident that mucosal cells are much more than a simple 'passive filter' and, through highly complex interactions, play a major role in regulating bioavailability of nutrients, drugs, and food contaminants. It is now clear that nutrients and contaminants present in the GIT influence expression and/or activity of some key proteins involved in regulation of cell growth, differentiation and apoptosis, with clear relations to pathologies such as cancer or intestinal inflammation.

Intestinal targets of food contaminants are cytochromes P450 (CYPs), conjugation enzymes and efflux pumps.

Interactions of CYPs with active ingredients of food supplements are developed later in this study. The activity of CYPs, which are proteins involved in metabolism of various drugs, may be modulated also by different food contaminants, such as those we have analyzed in the previous chapters. For example, some mycotoxins undergo metabolism by CYPs. In liver, CYP3A and CYP1A subfamilies have been shown to be involved in activation of mycotoxins, particularly AFB1 and ochratoxin A (OTA) related carcinogenesis (Sergent, et al., 2008), whereas few data relate to the interactions between mycotoxins and intestinal CYPs. Heavy metals such as lead, cadmium and mercury, and other toxic elements such as arsenic have been shown to be involved in CYP function and regulation (Sergent, et al., 2008). Combined effects of AhR ligands (PAHs) and heavy metals, frequently environmental co-contaminants, have been reported: studies in cell lines from humans, rodents, chicken and fish generally indicate that heavy metals diminish the inductive potency of PAHs for the CYP1A1, 1A2 and 1B1 and, therefore, could diminish the carcinogenicity of PAHs (Sergent, et al., 2008).

Efflux transporters play a key role in disposition and excretion of many substances, including endogenous metabolites, pharmacological drugs, as well as dietary compounds and various contaminants. Intestinal P-glycoprotein (Pgp) is a versatile multi-substrate pump, which can expel compounds out of the cells in order to return them to the luminal side of the intestinal barrier and maintain intracellular concentrations at low levels. The role of Pgp in drug interactions and bioavailability has been highlighted. Environmental contaminants such as pesticides, mycotoxins, antifungals and PAHs may modify the activity and expression of efflux pumps, such as the P-glycoprotein (Sergent, et al., 2008).

2.4 Analysis of active ingredients in selected food supplements

2.4.1 Identification of active ingredients in herbal food supplements

The six herbal plants categories chosen for our study all have well studied biological effects that were observed in the past.

- *Ginkgo biloba*: improve blood circulation and cerebral oxygenation
- St-John's wort (*Hypericum perforatum*): for mild depression
- Soy isoflavones (from *Glycine max*): reduce menopause effects
- Maca (*Lepidium meyenii*): increase libido and limit sexual disorders
- Black radish (*Raphanus sativus*): for bile secretion and intestine activity

- Garlic (*Allium sativum*): decrease arterial tension

It is important to note that natural products, unlike conventional drugs, contain a complex mixture of bioactive entities which may not all provide therapeutic activities. Often, a complete characterization of all the chemical constituents from a natural product is not available.

Additionally, chemical makeup of a natural product may vary depending on the part of the plant processed (stems, leaves, roots), seasonality and growing conditions (Chavez et al. 2005). Combination products composed of multiple natural products complicate matters further. In this study, we decided to only select products made from one plant species.

The active ingredients were measured for three categories of food supplements: *Ginkgo biloba*, Black radish and Maca.

2.4.2 Maca (Lepidium meyenii) active ingredients

Active ingredients: Lepidilin A, lepidilin B, macaridin and MTCA.

Important remark: In Maca, those alkaloïds may be considered as important specific active ingredients, but there is still no consensus. Nevertheless, they can be used as markers of authenticity. It seems, however, that some of the alkaloids are characterized by deleterious biological properties, such as carcinogenicity.

Some countries considers that consumption of MACA presents a risk for the consumer. For example (cited in AFSSA, 2004) :

The Danish authorities have considered that Maca root extracts present a risk for the consumers, they suspended it's commercialization in food supplements because of the following reasons:

- *The dried root powder cannot be considered as a common consumed plant in Europe;*
- *The presence of alkaloids in the Maca root;*
- *The doses inducing effects on the sexual behavior of rats (0,15 to 75 mg/kg body weight) are in the range of those advised by the providers of the food supplements (around 67 mg/kg bw).*

Detailed methodology and results of the analysis of MACA are available in the final report of phase I of the present project (Mormont et al, 2009).

Analysis of six maca products (3 notified, 3 not notified) by nano-LC-MS revealed one product containing no lepidilin A and no lepidilin B (see Table V). From these data, it could be concluded that this sample does not resemble a maca-extract. Nevertheless, macaridin was detected in all maca supplements, with no correlation with the presence of lepidilins. A small amount of MTCA ((1R,3S)-1-methyltetrahydro-ß-carboline-3-carboxylic acid) has been detected in a notified sample. This alkaloid is

considered as a mutagenic precursor responsible for neuronal death by the AFSSA (AFSSA, 2004). Nevertheless it must be noted that MTCA is found in many foods and that the mutagenicity appears to be largely linked to concomitant factors within the chosen experimental settings (Gonzales et al 2008).

TABLE V: Active ingredients of the six Maca products

Brand name	***Product name***	*Normalised area/ µg supplement*			
		Lepidilin A	*Lepidilin B*	*Macaridin*	*MTCA*
Biodynamics bvba	MACA 500	10	13	28	Not
Laboratoires Fenioux	Maca	5	14	100	Not
Decola (Vivadis)	Maca	9	14	16	< 20 µg/kg
Matisson Healthcare	Active maca	100	100	51	Not
AOV	MACA 500MG	37	24	29	Not
Dieti natura	Maca	Not	Not	51	Not

None of the six maca products announced the concentration of any potentially active ingredients on the label.

2.4.3 Black radish (Raphanus sativus) active ingredients

Active ingredients: glucosinolates (GL)

The glucosinolate (GL) profile in black radish (*Raphanus sativus)* based dietary products was investigated. An analytical strategy combining the use of LC-PDA, LC-ESI-MS/MS and LC-APCI-MS/MS systems was applied. The LC-ESI-MS/MS system was used to detect and identify the naturally occurring intact GLs. The identified intact GLs were then desulfated and quantified on an LC-PDA system as desulfo-GLs. Prior to quantification, the desulfo-GLs were identified using an APCI-MS/MS system. A detailed description of the analytical method can be found in Njumbe Ediage et al. (2011). In total, six glucosinolates were identified and determined (quantified) in the dietary products (see TABLE VI). The quantitative data revealed a great diversity in the individual GL content in the six dietary products. This variation of the total GL content can be attributed to differences between species and subspecies of the different black radishes from which the products were derived. Different growing conditions could also have contributed to the differences in the glucosinolate content in the different samples (Ciska et al. 2000).

TABLE VI: Glucosinolates content in the black radish products. rRF: response factors (ISO 9167−1−1992). The calculation of the intake/day took into account the recommended number of capsules, the amount of plant material per capsule and the total GL per capsule.

Brand name	Product name	Desulfoglucoraphenin	Desulfoglucoputrajivin	Desulfogluconasturtin	Desulfoglucosisaustricin	Desulfoglucoraphasatin	Desulfoglucosisymbrin	Total	Recommended intake/day
		(mg/g)							(mg)
	rRF	0.9	1	0.95	1	0.40	1.32		
ARKOPHARMA - BELUX	Arko Radis noir	1.01	0.24	0.06	0.50	0.32	0.73	2.85	2.052
EPHYTO	Radis noir	1.12	0.2	0.07	0.91	0.20	0.76	3.26	2.44
DIETI NATURA	Radis noir	1.27	0.28	0.12	0.45	0.48	0.99	3.59	4.45
LABORATOIRES FENIOULX SPRL	Radis noir	1.16	0.32	0.08	0.77	0.24	0.74	3.30	2.64
SUPER DIET	Radis noir	1.23	0.17	0.08	0.37	0.36	0.80	3.00	1.62
PIERRE FABRE SANTE BENELUX SA	Elusan Radis Noir	0.84	0.14	0.10	0.65	0.20	0.70	2.62	1.31

Glucoraphenin was the most abundant glucosinolate in all samples. Glucoraphasatin and glucoraphenin have been reported as the main GLs in radish roots (Montaut et al. 2010). These GLs were also confirmed in all six black radish based dietary products. Since glucoraphasatin and glucoraphenin differ only in the degree of oxidation of the sulfur atom in the side chain, direct biological reduction of glucoraphenin to glucoraphasatin during sprouting could explain the possible co−existence of both glucosinolates in radishes.

The daily intake of total GLs when following the advised intake varies from 1.31 -> 4.45 mg, which can be considered as a considerable variation.

None of the six black radish products presented as food supplements for the consumer announced on the label the concentration of any potentially active ingredients.

2.4.4 St John's wort (Hypericum perforatum) active ingredients

Active ingredients: hyperforin, hypericin

Initially, hypericin was considered to be the antidepressant constituent of St John's wort (SJW). This is the reason why only the hypericin content is obligatory to mention on the label. In the last decade, experimental and clinical evidence has shown that hyperforin is a major constituent required for the antidepressant activity. Many products containing extracts of SJW are still standardized on hypericin content because hyperforin is thought to be unstable (Barnes, 2002). None of the products bought refers to hyperforin content.

Moreover, recent interest in St John's wort concerns mounting evidence that the component hypericin may be associated with cataractogenesis. Some details about this hypothesis are given in chapter 2.6.1.

The Royal Decree of 1997 (MB, 1997) mentions that "the daily absorption recommended on the packaging or in the advertisement can't have the consequence of hypericin consumption higher than 700 µg per day". Nevertheless, for the majority of the products, when the hypericin concentration was available, we observed that the consumption of only one pill was sufficient to exceed those 700 µg. Those five products are not notified (see right column in TABLE VII).

We also observed the lack of hypericin content information on two notified food supplements.

TABLE VII: Hypericin content specified on the packaging. FS: Food supplements (notified products), MP: medicinal products, NNP: not notified products. Cells in red=issues.

Brand name	***Product name***	***Type***	***Hypericin content on the label***	***Hypericyn/cap*** *(mg/cap or tab)*	***Hypericyn/day*** *(mg/day)*
PARABOLIC BIOLOGICAL SPRL	SINT-JANSKRUID STERK	FS	hypericin and pseudohypericin: 0.3 mg/243 mg extract	0,30	0,60
LICHTWER PHARMA BENELUX (Regipharm nv)	Kira® Sint Janskruid LI 160	MP	Not found	-	-
SPRINGFIELD	ST JANSKRUID 500MG	NNP	hypericin (0,3%)= 1.5 mg/caps	1,50	2,25
SOLGAR VITAMINS	St John's Wort Herb 300 mg Extract	NNP	hypericin 0,3% of extract (300mg)	1,00	1,00
VITAMIN HEALTH	Goed Gemoed, Sint Jans Kruid	NNP	hypericin 0,3% of extract (300mg)	1,00	1,00
SOURCE NATURALS	St. Johns Wort, st Jans Kruid	NNP	hypericin 0,3% of extract (300mg)	1,00	3,00
HAGOR BIOSERVICE NV	Bakanasan Huile de Millepertuis	NNP	Not found	-	-
ARKOPHARMA - BELUX	Arkogélules Millepertuis	FS	Not found	-	-
LABORATOIRES FENIOULX SPRL	MILLEPERTUIS FORT	FS	Not found	-	-
DIETI NATURA	Millepertuis	NNP	Not found	-	-
MAXIMUM NUTRIENTS	St John's Wort 60	NNP	hypericin 0,3% of extract (300mg)	1,00	2,00
NATROL	Millepertuis	NNP	Not visible (package damaged)	N/A	-
VITADYNE	Millepertuis	MP	hypericin standardized (mcg)= 233µg/caps	0,23	0,69

The determination of the active ingredients hyperforin and hypericin in St John's wort based food supplements was performed using high performance liquid chromatography coupled to an ultraviolet, diode array and fluorescence detector (HPLC-UV/FLD). The analytical method was developed using commercial hyperforin and hypericin standard solutions and applied to the selected St John's wort samples for the determination and quantification of these active compounds.

The results for the determination of hyperforin and hypericin in 12 St. John's Wort food supplements can be found in Table VIII.

In the literature, some authors developed different methods of liquid chromatography for the determination of the active compounds of St. John's Wort dietary supplements. The concentration for hypericin ranged between 0.1 and 0.9 mg/g dry powder (Liu *et al.,* 2000; Ganzera *et al*. 2002). The concentration detected for hyperforin ranged between 2.4 and 9.3 mg/g of product (Liu *et al.,* 2000) or between 0.4 and 13 mg/g (Ganzera *et al*. 2002). Studies of St. John's Wort products (tablets and capsules containing *Hypericum perforatum* extract or plant material) showed significant differences in their qualitative and quantitative composition. This variation was explained by the instability of hyperforin (thermolabile and photosensitive).

Our results are in accordance with those results found in the literature: hypericin concentration ranged between 0.02 and 0.8 mg/g and was detected in all the samples The concentrations detected for hyperforin were between 1.3 and 36 mg/g.

Table VIII: Results of the hypericin/hyperforin analysis in St. John's Wort food supplements.

Name	Amount of substance per caps/weight of tablet (mg)	Recommended ingestion per day (Caps/Tablets)	Hyperforin (mg/g)	Hypericin (mg/g)	Hyperforin (mg/caps or tab)	Hypericin (mg/caps or tab)
SINT-JANSKRUID STERK	410	2 caps	1.30	0.02	0.53	0.01
Kira® Sint Janskruid LI 160	750	1-2 tablets	36.17	0.32	27.12	0.24
ST JANSKRUID 500MG	500	1-2 caps	36.59	0.34	18.29	0.17
St John's Wort Herb 300 mg Extract	460	1 caps	22.19	0.05	10.21	0.02
Goed Gemoed, Sint Jans Kruid	515	1 caps	16.91	0.43	8.71	0.22
St. Johns Wort, st Jans Kruid	480	3 tablets	28.09	0.74	13.48	0.35
Arkogélules Millepertuis	345	1 caps	13.13	0.44	4.53	0.15
MILLEPERTUIS FORT	190	3-6 caps	4.48	0.27	0.85	0.05
Millepertuis	260	4-6 caps	12.62	0.78	3.28	0.20
St John's Wort 60	470	1-3 caps	13.35	0.33	6.27	0.16
Millepertuis	1050	Not found	12.88	0.20	13.53	0.21
Millepertuis	440	3 caps	5.28	0.02	2.32	0.01

ND: not detected

2.4.5 Ginkgo biloba active ingredients

Active ingredients: Bilobalide, ginkgolide, flavonols (kaempferol, quercetin, isorhamnetin).

Only three products were analyzed in order to quantify their flavonol glycosides (1 FS, 1 NNP, and 1 MP). Results are compared to the expected concentrations (see TABLE IX), and show some discrepancies, which are difficult to explain because of the limited number of samples analyzed.

TABLE IX : Expected and measured concentrations of flavonol glycosides in three *Ginkgo biloba* products. FS: Food supplements (notified products), MP: medicinal products, NNP: not notified products.

Product name	***Type***	Ginkgo extract	Expected flavonol concentration	Sample weight (mg)	Measured flavonol glycoside	
		mg/tab	(mg/tab)	mg	mg/100mg	mg/tab
Ginkgo	NNP	40	10	316	17	53
Bio-Biloba	MP	100	24	427	26	82
Ginkgo biloba	FS	110	12	363	11	36

Conclusions from the analysis of active ingredients

The analysis of active ingredients in the selected products shows that the content of the product is not always mentioned on the label. This is mandatory, according to the European Directive 2002/46/CE, transposed in Belgian legislation in the "plant" Royal Decree of 1997. But, as this "plant" decree is not yet complete, because the active ingredients are not identified for each plant food supplement, this obligation is not applicable.

This shows the need for more research to identify active ingredients in plant FS as well as a gap to fill in the European and Belgian legislation.

2.5 *In vitro* studies of active ingredients and food supplements extracts

2.5.1 General approach

In phase I and II of this project, several relevant *in vitro* studies were performed by the different partners. The different *in vitro* techniques were applied to obtain information on:

- ✓ The individual food supplement active ingredients. Therefore *in vitro* studies were performed with the individual active ingredients obtained as pure standards;
- ✓ Possible interactions between the active ingredients. Mixtures of active ingredients from one food supplement were analyzed at concentrations similar to those present in a standardized extract (reference material from NIST);
- ✓ Food supplement extracts. The same *in vitro* tests were performed with a standardized extract (reference material from NIST).

The constraints were already mentioned during the first phase of the project: standards molecules are not available for all the active ingredients concerned, some ingredients are unstable, and care must be taken to avoid the influence of the conservation duration.

Because of these constraints, as well as time and budget constraints, we have decided to focus on 3 categories of products, on the basis of the frequency of their consumption : soy isoflavones, St John Wort and *Ginkgo biloba*. For these 3 categories of products, we have tested their active ingredients separately with our panel of *in vitro* tests, as pure standards. The complete study (*in vitro* testing of active ingredients separately, mixture of active ingredients and plant extract) has been performed only on *Ginkgo biloba*. We have not analyzed real samples at this stage, we rather preferred to analyze a reference material (from the National Institute of Standardization, NIST), with certified concentration of active ingredients of *Ginkgo biloba*.

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 has been 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.

2.5.2 Overview of the in vitro assays

2.5.2.1 General toxicity of the active ingredients

The toxicity of active ingredients was evaluated using bacteria (*E.coli*) and eukaryotic cells (HepG2 and Caco-2). For Caco-2 cells, two types of cytotoxicity assays were performed. First, inhibition of cell proliferation by active ingredients of food supplements was tested with the colorimetric MTS assay. Second, the release of lactate dehydrogenase (LDH) activity in the culture medium by necrotic cells was measured after incubation of 16-day post-confluent cells with active ingredients during 6h or 24h, before measuring CYP1A1 activity in cells (see below). The LDH assay constitutes a preliminary test, to ensure that concentrations of active ingredients used to measure CYP1A1 activity are not toxic for cells.

2.5.2.2 Toxic mode of action

Possible targets of active ingredients were identified by analyzing the effect on the expression of 14 genes involved in four different modes of action (oxidative damage, DNA damage, general cell lesions and membrane damage). They were performed with a bacterial reporter assay, carrying different stress promoters fused to the lac Z gene. The induction of the different stress genes was measured through the β-galactosidase activity. The index of the 14 genes and their function is shown in Table X (Dardenne et al., 2008).

TABLE X: Stress gene promoters fused to the *lacZ* reporter gene and their major inducers.

Promoter	Gene product/Function	Responsive to
KatG	Hydrogen peroxidase I	Oxidative stress
MicF	Antisense RNA to 5' OmpF	Membrane integrity, osmotic stress
OsmY	Periplasmic	Protein Osmotic stress
UspA	Universal stress protein	Growth arrest
RecA	General recombination and DNA repair	SOS response
Zwf	Glucose-6-phosphate dehydrogenase	Oxidative stress
ClpB	Proteolytic activation of ClpP	Protein perturbation
UmuDC	DNA repair	Radiation and/or chemically induced DNA damage
MerR	Regulation of the mercury resistance operon (mer)	Heavy metals
Ada	Adaptive response to alkylation	DNA damage, mainly methyl adducts
DinD	Unknown function within the DNA damage inducible response	DNA damage
Soi28	Superoxide inducible gene	Superoxide radical generating agents
Nfo	Endonuclease IV	Single-stranded and double-stranded DNA breaks, oxidative DNA damage
SfiA	Inhibitor of cell division	SOS response

2.5.2.3 Hormonal and dioxin-like activities

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 response sequence of DNA, a promoter and the firefly 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 test activation of the Aryl hydrocarbon receptor (AhR) and the second group responds to activation of the different specific steroid hormone receptors. The principle of reporter gene assays is shown in Figure 8.

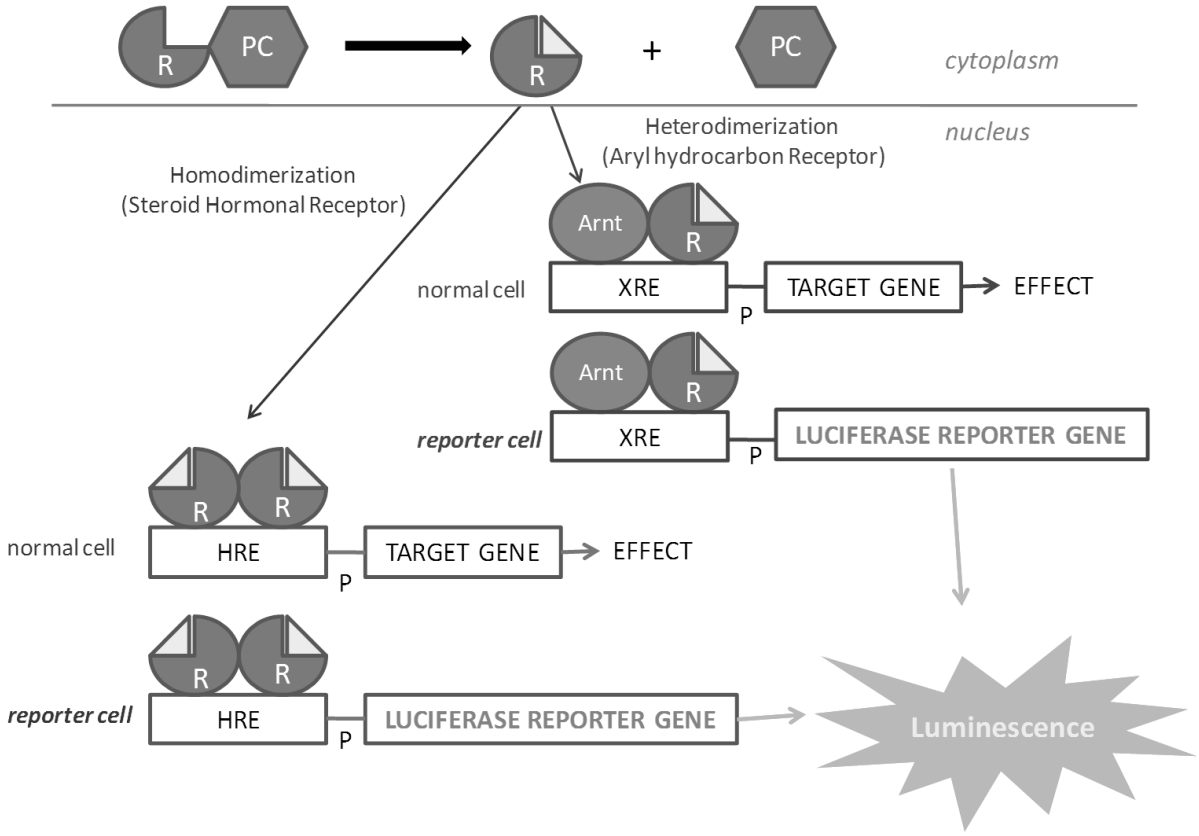


Figure 8: principle of reporter gene assays taking advantage of the AhR and steroid receptors pathways. R= receptor, PC= protein complex, XRE= Xenobiotic Responsive Element, HRE= Hormone responsive Element, P= promoter, Arnt= Aryl receptor nuclear translocator. Yellow triangle is the ligand.

The AhR and the steroid hormone receptor pathways are very similar. Briefly, the inactive receptor is located in the cytoplasm within a protein complex. Upon binding of the ligand, the receptor crosses the nuclear 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 8). In normal cells, target genes are transcribed and different effects occur in the cells. In reporter cells, the product of the additional luciferase reporter gene produces a luminescence, after addition of substrates, which is directly correlated with the ligand concentration (Scippo *et al.,* 2004; Van der Heiden, 2009, Willemsen *et al.*, 2002, 2004 and 2005).

The evaluation of the **dioxin-like or anti-dioxin activity** of active ingredients was performed by studying the capacity of the active ingredient to activate (agonistic effect) or to inhibit the activation (antagonistic effect) of the aryl hydrocarbon receptor (AhR). For this, we used the luciferase reporter cell lines H4IIE-XRE, which are derived from rat hepatoma cells and thus express the rat Ah receptor (rAhR), and HepG2-XRE, which are from human hepatoma cells and thus harbor the human Ah receptor (hAhR). These cells were exposed to one active ingredient (**agonistic assays**) or to a mixture of the reference AhR ligand and each active ingredient (**antagonistic assays**).

Let's note that the rat cell line is more sensitive to dioxin than the human one. The concentration of half-maximal response (EC50) of TCDD for the rat and human hepatoma cell lines are 48 pM and 800 pM (24h) for H4IIE-XRE and HepG2-XRE, respectively, showing a lower sensitivity of HepG2-XRE cells towards TCDD, probably resulting from the different species origin of the Ah receptor.

As the major target of the AhR transcription factor is the CYP1A1 metabolization enzyme, an effect of a tested compound observed on activation or inhibition of the AhR will indicate that this compound probably also interacts with CYP1A1 expression in liver.

To study the hormonal or anti-homonal activity of active ingredients, we used reporter cells derived from human mammary gland carcinoma, genetically modified to contain the firefly luciferase gene under a control of an inducible promoter. Four cell lines were used, sensitive to

- ✓ Estrogen hormones (MCF-7 derived cells, containing mainly the human estrogen receptor alpha, hERalpha),
- ✓ Androgen hormones (T47-D derived cells, containing the human androgen receptor, hAR),
- ✓ Progestagen hormones (T47-D derived cells, containing the human progestagen receptor, hPR),
- ✓ Glucocorticoid hormones (T47-D derived cells, containing the human glucocorticoid receptor, hPR).

As most of the known endocrine disruptors display their effect by acting on a steroid receptor (mostly hER or hAR), an effect (activation or inhibition) on one or more steroid receptors of a tested compound indicates that the compound is a potential endocrine disruptor.

2.5.2.4 Effect on human cytochromes P450 (CYP) 1A1 and 3A4 activities in human colon adenocarcinoma cells (Caco-2 cells).

In this study, we used the human colon adenocarcinoma cell line Caco-2 (ATCC, Rockville, MD), a well-known *in vitro* model of the intestinal barrier.

CYP1A1 brief definition: CYP1A1 is an inducible cytochrome P-450 present in intestinal cells and other tissues such as liver. Although most CYP oxidations are detoxification pathways, the chemistry of certain substrates leads to the production of reactive cytotoxic metabolites. For example, procarcinogens such as polycyclic aromatic hydrocarbons (PAHs), nitrosamines and food-derived aromatic amines may be activated to DNA-binding species by CYP1A1. Any compound able to increase the CYP1A1 activity is potentially dangerous for the organism.

Different interactions were analyzed using the 7-ethoxyresorufin dealkylase (EROD) assay:

- The inductive effect on CYP1A1 activity in presence of the active ingredients only.
- The inhibitory effect on the induction of CYP1A1 activity by benzo[a]pyrene (B[a]P), a well-known CYP1A1 inducer in enterocytes and Caco-2 cells.

CYP3A4 brief definition: Cytochrome P-450 3A4 (CYP3A4) is the predominant CYP isoform in the small intestine. It contributes to 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. Therefore, a modification in the CYP3A4 activity may modulate the bioavailability of some drugs, whith consequences more or less important for the patient.

The activity of the CYP3A4 cytochrome can be induced or inhibited by a number of usual foods. As strange as it appears, the best documented one is grapefruit juice. A grapefruit juice-drug interaction was first accidentally reported in 1991 (Bailey and Spence, 1991) when used as a flavor supplement to mask the taste of ethanol during a study of ethanol and felodipine interaction. A single glass of grapefruit juice caused a two- to three-fold increase in felodipine plasma levels compared to orange juice, which had no effect. It was observed that grapefruit juice inhibits the CYP3A4 activity, which leads to the increased blood level of the drug. Since then, many other drugs have been shown to have their bioavailability increased by grapefruit juice, such as cyclosporine, fenadine, midazolam and lovastatin. This excessive level of drug in the blood can lead to toxic levels, to the extent of causing an overdose. The onset of interaction can occur within 30 minutes

following intake of a single glass of grapefruit juice, and the inhibition can last up to 3 days.

Food supplements are well known to influence activity of the CYP3A4 protein. Because of the exponential increase of food supplement consumption, it is important to study their possible interactions with CYP3A4 activity in order to better understand the positive influence on cytochrome activity.

Different interactions were analyzed by measuring the conversion of testosterone to 6β-(OH)-testosterone by the CYP3A4 isoform and detection of the metabolite by HPLC:

- The inductive effect on CYP3A4 activity after a treatment of two weeks with the active ingredients. Cells are thereafter exposed to testosterone during 3h.
- The inhibitory effect on CYP3A4 activity after pre-treatment with 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3), a CYP3A4 inducer. Cells are thereafter exposed to both testosterone and the active ingredients during 3h.

More details on the three kinds of *in vitro* assays are available in the final report of phase I of the present project (Mormont et al, 2009).

This kind of approach, based on complementary *in vitro* techniques used as a "tool box", is relevant to characterize the possible biological effects of a compound or a mixture of compounds (Ribonnet et al, 2011), but it is obvious that possible effects evidenced *in vitro* have to be confirmed *in vivo*, and, if possible of course, in humans.

2.5.3 In vitro studies of active ingredients from Black radish

Effects of active ingredients of Black radish on targeted stress genes

The concentrations used in this study were calculated on the basis of growth inhibition experiments. The appropriate concentration ranges were selected. Results are shown on TABLE XI.

The enantiomers of sulforaphane that were tested show clear and remarkable differences in especially the level of induction of MerR, 2.19 for DL-sulforaphane and 72.1 for L-sulforaphane. MerR has a human analogue: MT. 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. On the other hand, the affinity of the methalothionein to some useful metals might reduce their bioavailability and thus provoke a deficiency.

At this point no conclusion can be given on whether or not the activation of a metallothionein is positive for health. Nevertheless, the Black radish food supplements are taken for their action on the intestinal tract (for example bile secretion), not a hypothetical heavy metal detoxification activity. Regarding this, the induction of the MerR gene expression by glucosinolates (which are also found in common food such as broccoli, Brussels sprouts and other cruciferous vegetables) should be further investigated.

TABLE XI: *E.coli* induced stress genes by L- and DL-Sulphoraphane. -: no significant induction.

	***E.coli* significant induced genes**			
Active ingredients *(concentrations tested)*	***oxidative damage*** *KatG, Zwf, Soi28, Nfo, MerR*	***membrane damage*** *MicF, OsmY*	***cellular stress*** *UspA, ClpB*	***DNA damage*** *RecA, UmuDC, Ada, DinD, SfiA*
L-sulforaphane (0,2 -> 12,5 µM)	KatG (1,2), MerR (72,1)	OsmY (2,1)	-	DinD (2,2)
DL-sulforaphane (1 -> 60 µM)	KatG (1,3), MerR (2,2)	OsmY (2,9)	ClpB (1,3)	DinD (2,2)

2.5.4 In vitro studies of active ingredients from St John' wort (Hypericum perforatum L.)

Hypericin and hyperforin standards, the two major relevant ingredients of St John's wort, were used to perform different *in vitro* assays. The working concentrations have been calculated on the basis of the packaging information (concentration of standardized active ingredients and recommended daily intake) and the information from literature (measured concentration of non-standardized active ingredients), both converted in plausible concentration in the intestine (dilution hypothesis of the estimated daily intake in 1l of gastro-intestinal fluid). The plausible concentrations are:

- Hypericin: 0,5ppm->5ppm
- Hyperforin: 10ppm->100ppm

In our *in vitro* assays, these concentrations are plausible when Caco-2 cells (intestinal cells) are exposed to the compounds. For other cell systems (liver or mammary gland), it is not possible to calculate such plausible concentrations, but without other available information, these intestinal plausible concentrations were

used as reference concentrations for the other cell systems as well. This is true for all the active ingredients tested in this study.

2.5.4.1 General toxicity of the active ingredients of St John' wort

The results of the general toxicity study of hyperforin and hypericin are presented in **TABLE** XII.

TABLE XII: General toxicity and inhibition of cell proliferation by hypericin and hyperforin. IC50: concentration at which 50% of the growth is inhibited, LOEC: Lowest Observed Effect Concentration, LC50: Lethal Concentration at which 50% of the cells are dead.

	Bacteria		*Eucaryotic cells*			
Active ingredients (plausible concentration)	***E. coli growth inhibition (IC 50)***	***E.coli growth inhibition (LOEC)***	***Cytotoxicity in HepG2 cells (LC50)***	***Cytotoxicity in HepG2 cells (LOEC)***	***Caco 2 cells proliferation (MTS assay) (LOEC)***	***Cytotoxicity on Caco 2 cells (LDH assay)***
Hypericin (0,5 ppm -> 5 ppm)	38,3 ppm	12,5 ppm	15,5 ppm	12,5 ppm	12,5 ppm	no effect
Hyperforin (10 ppm -> 100 ppm)	>10 ppm	> 10 ppm	**1,3 ppm**	**1,3 ppm**	**1,5 ppm**	no effect

Results obtained using bacteria and eukaryotic cells are highly difficult to compare since the biological materials are different. There seems to be no link between the bacterial and the eukaryotic reactions to hypericin and hyperforin. Nevertheless, the LOEC value on human HepG2 cells for hypericin is ten times higher than for hyperforin, and also far higher than the plausible concentration in the intestine. This is also observed on human HepG2 for the LC50 (Lethal Dose at which 50% of the cells are dead). No harm would be expected thus from hypericin. In contrast, the much lower LOEC and LC50 concentrations observed for hyperforin are dramatically lower then its plausible concentrations, except for cytotoxicity in Caco-2 cells. This indicates that hyperforin is highly toxic for HepG2 cells and could provoke damages in the liver. In human Caco-2 cells, no LDH release (indicator of cell necrosis) was observed, but just the inhibition of cell proliferation.

A possible explanation could be that the toxicity is provoked by some metabolites from hyperforin only secreted in liver cells and not in Caco-2 cell, but it is not well established if and how caco2 cells can metabolise certain compounds. These cell type specific effects could be further investigated.

2.5.4.2 Effects of active ingredients of St John' wort on targeted stress genes

Neither hypericin nor hyperforin induced expression of any of the 14 screened stress reporter genes (data not shown).

The known mode of action of St-John's wort - one of the most frequently studied medicinal plants - is mainly based on hyperforin. Its major pharmacological

activities affect unique eukaryotic systems such as neurotransmitter reuptake inhibition, CYP1A1 inhibition, anti-tumoral properties, thus all specific modes of action that cannot be evaluated using a bacterial system.

2.5.4.3 Dioxin-like activity of the active ingredients of St John' wort

Agonistic assays

The cells were exposed to each standard active ingredient during 6h and 24h to assess the dioxin-like activity (agonistic activity). No dioxin-like activity was observed, neither on rat nor on human hepatoma cells (results not shown).

Antagonistic assays

Antagonistic assays were carried out to measure a possible inhibition of the AhR pathway by standard active ingredients, when cells are co-exposed to AhR reference ligand, the TCDD (tetrachlorodibenzodioxin). Concentrations of TCDD were applied to the cells to induce 50% of the maximal inducible luciferase activity (EC50): 50 pM for H4IIE-XRE cells and 0.8 nM for HepG2-XRE. At the same time, increasing concentrations of standard active ingredients were added to the cells. The cells were exposed to those ingredients during 6h and 24h to assess the influence of the time exposure. Results are presented on **Table** XIII.

When rat hepatoma cells H4IIE-XRE are co-exposed, during 6h or 24h, to TCDD and hyperforin or to TCDD and hypericin, they generate a higher luciferase activity than when exposed to TCDD alone. Thus we observed a synergy between TCDD and hypericin (see also Figure 9 for hyperforin results), because no response was observed when these compounds were applied alone on the cells (see above).

Table XIII: antagonistic activities of hyperforin and hypericin in dioxin-sensitive cells co-exposed, during 6h or 24h, to TCDD and hyperforin or hyperici.
-: no activity; +: antagonistic activity (response < 75% of the TCDD half maximal response); Synergy: response > 100% of the TCDD half maximal response. Number in brackets : minimal concentration (ppm) showing an inhibition (corresponding to 75% of the TCDD half maximal response) of the Ah receptor. PC : plausible concentration.

Active ingredients (PC in intestine)	*inhibition of rAhR (6h or 24h cell exposure)*	*inhibition of hAhR (6h or 24h cell exposure)*
	ppm	*ppm*
Hypericin (0,5 - 5 ppm)	synergy "TCDD-hyperforin" (0,3)	+ (1,3)
Hyperforin (10 - 100 ppm)	synergy "TCDD-hyperforin" (0,3)	+ (0,5)

In contrast, in human hepatoma cells HepG2-XRE, both hyperforin and hypericin seemed to display antagonistic activities when exposed to the cells during 6h or 24h (figure 10, illustrated for hyperforine). We observed some negative relative

responses for the highest tested concentration but MTT tests results indicated good cell viability (data not shown).

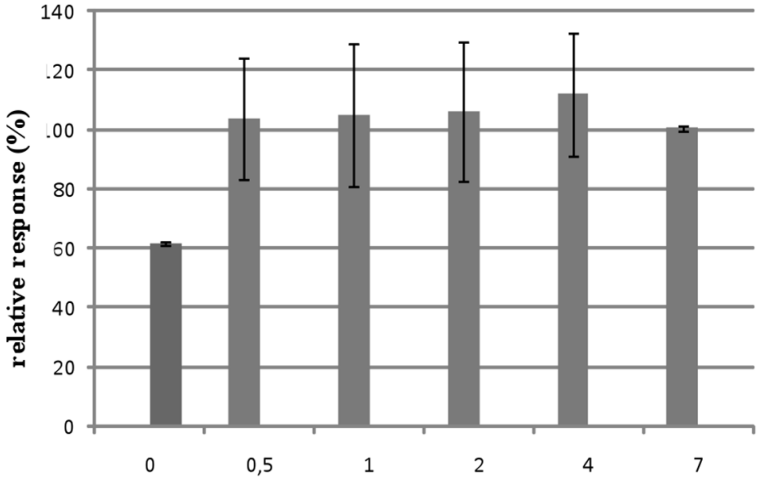


Figure 9: Synergy between hyperforin and TCDD in rat hepatoma cells H4IIE-XRE exposed to a mixture of TCDD (50 pM) and increasing concentrations of hyperforin (exposure time of 24h).

Those results suggest that both hypericyn and hyperforin may modulate the cellular response to dioxin compounds and the AhR pathway. Interestingly, this modulation is different in rat (synergy) and human (inhibition) cells.

As it is well known that the main target gene of the AhR is the CYP1A1, we can conclude that both hypericin and hypericin may modulate CYP1A1 activity in liver cells.

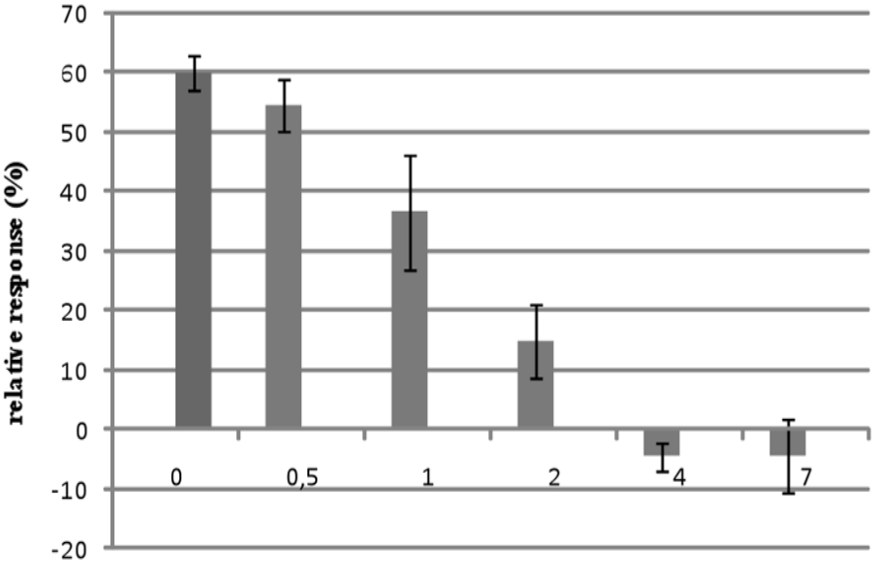


Figure 10: Antagonistic activity of hyperforin in human hepatoma cells HepG2-XRE exposed to a mixture of TCDD (50 pM) and increasing concentrations of hyperforin (exposure time of 24h).

2.5.4.4 Hormonal-like activity of the active ingredients of St John' wort

Agonistic assays

Estrogen-sensitive cells were exposed during 24h to each standard active ingredient while the three other hormone-sensitive cells (cells sensitive to androgen, progestagen and glucocorticoids) were exposed during 48h, in order to assess the hormonal-like activities (agonistic activities) of hyperforine and hypericine in a range of concentrations including the plausible concentrations.

No hormone-like activity was observed neither for hypericin nor for hyperforin (results not shown).

Antagonistic assays

Antagonistic assays were carried out to measure a possible inhibition of the steroid receptor pathways by hyperforin or hypericin when cells were co-exposed to the steroid receptor ligand (= inducer). Concentrations of inducer were applied to the cells to induce 50% of the maximal inducible luciferase activity (=EC50). The results were compared to the maximal response obtained with the reference ligand for each steroid receptor (hAR: 5 alpha-dehydrotestosterone, hPR: progesterone, hGR: dexamethasome and hER: 17beta-estradiol) and the outcomes of two independent experiments are shown in the Table XIV.

Table XIV: antagonistic activities of hypericin and hyperforin in hormonal-sensitive cells co-exposed to hypericin or hyperforin and the steroid receptor reference ligand (see above).
-: no activity; +: antagonistic activity (response < 75% of the TCDD maximal response); /: not tested. Number in brackets : minimal concentration (ppm) showing an inhibition (corresponding to 75% of the reference ligand half maximal response) of the steroid receptor. PC : plausible concentration.*: visual cytotoxicity observed by microscopic examination of the cells.

	INHIBITION			
Active ingredients (PC in intestine)	***human AR***	***human PR***	***human GR***	***human ER***
Hypericin (0,5 - 5 ppm)	*	/	+ (5,0 ppm)	+ (1,3 ppm)
Hyperforin (10 - 100 ppm)	*	/	*	+ (0,5 ppm)

In oestrogen sensitive cells, both hyperforin and hypericin seem to display hER antagonistic activities We observed some negative relative responses for hyperforin for the highest tested concentrations (4 and 7 µM) (Figure 11), but MTT test results indicated good cell viability (data not shown). In glucocorticoid sensitive cells, only hypericin exerted antagonistic activities (data not shown).

These results show that both hypericin and hyperforin display anti-estrogenic activity *in vitro*, at the plausible intestinal concentrations taken here as references.

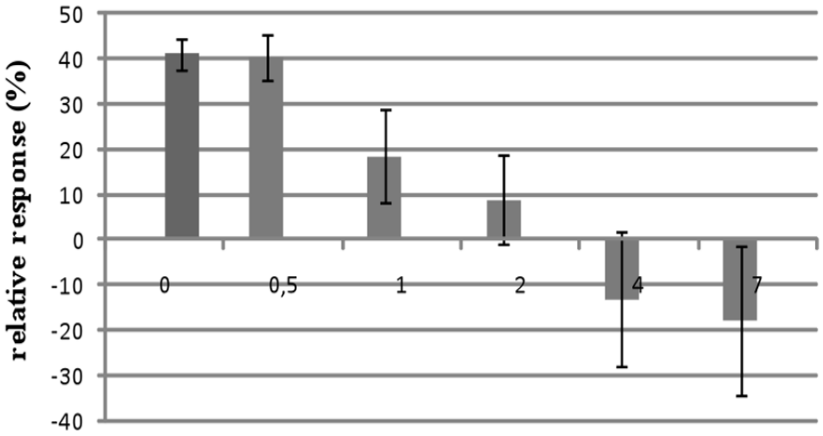


Figure 11: antagonistic activity of hyperforin in human breast tumour cells MCF7-ERE exposed to a mixture of 17beta-estradiol (12.5 pM) and increasing concentrations of hyperforin (exposure time of 24h).

2.5.4.5 Interactions of the active ingredients of St John' wort with the cytochrome CYP1A1 on Caco-2 cells

Caco-2 cells have been exposed during 6h or 24h to active ingredients of St John' wort (hypericin or hyperforin), in presence or not of B[a]P, a well-known contaminant inducing CYP1A1 activity.

Results are presented on TABLE XV, Figure 12 and Figure 13.

Table XV: Impact of pure compounds as standards on CYP1A1 activity in presence or absence of B[a]P, in 16-day post-confluent Caco-2 cells. -: no effect.

	Concentrations tested (ppm)	***Impact on CYP1A1 activity*** *6h and 24h exposure on 16-day post-confluent cells*	***Impact on B[a]P- induced CYP1A1 activity*** *6h exposure on 16-day post-confluent cells*
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

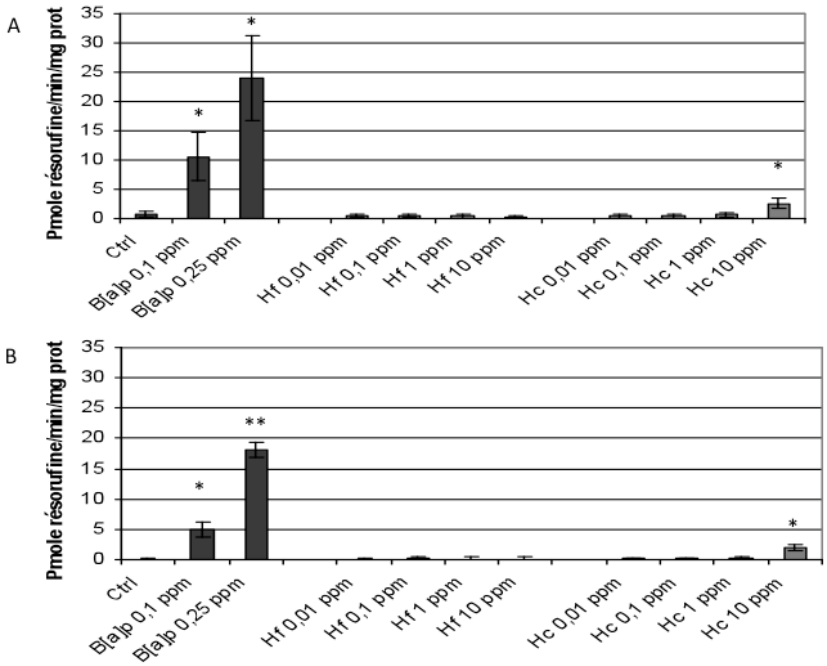


Figure 12: Induction of CYP1A1 activity in Caco-2 cells after 6h (A) or 24h (B) exposure to different concentrations of hyperforin (Hf) or hypericin (Hc). Sixteen-day post-confluent cells seeded in 48-well plates (4.10^4 cells/cm^2) were exposed to 0.01, 0.1, 1 or 10 ppm of Hf or Hc or to controls for 6h or 24h. The negative control consisted of FBS-free culture medium alone and the positive control in cells treated with B[a]P (0.1 or 0.25 ppm). CYP1A1 activity was assessed through the measurement of the fluorescence produced by the conversion of 5 μM 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin produced/min/mg protein. Values are means ± S.E.M. of three independent experiments performed in triplicates (n=9).* p<0.05, ** p<0.01, as compared to negative control.

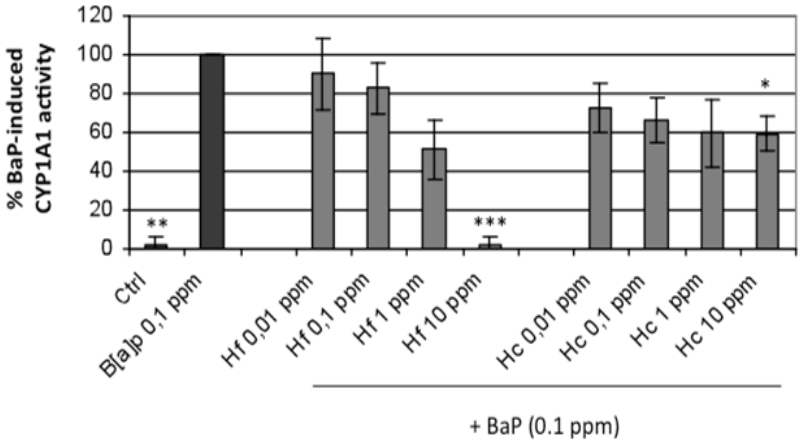


Figure 13: Decreasing effect of different concentrations of hyperforin (Hf) or hypericin (Hc) on B[a]P-induced CYP1A1 activity in Caco-2 cells. Sixteen-day post-confluent cells seeded in 48-well plates (4.10^4 cells/cm^2) were exposed to 0.1 ppm of B[a]P alone or simultaneously with 0.01, 0.1, 1 or 10 ppm of Hf or Hc for 6h. The control (Ctrl) consisted of FBS-free culture medium alone. CYP1A1 activity was assessed through the measurement of the fluorescence produced by the conversion of 5 μM 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin produced/min/mg protein and presented in % of B[a]P-induced CYP1A1 activity. Values are means ± S.E.M. of three independent experiments performed in triplicates (n=9).* p<0.05, ** p<0.01, *** p<0.005 as compared to negative control.

Only **hypericin** seems to increase CYP1A1 activity at a concentration of 10 ppm. Nevertheless, this increase is weak compared to the activity induced by B[a]P. *In vivo*, the plausible concentrations of hypericin (from 0.5 ppm to 5 ppm) are slightly lower to those required to observe this reaction in the *in vitro* model, suggesting that the intake of plausible doses of St John's wort may slightly increase CYP1A1 activity.

After co-exposure with **hypericin**, a slight decrease of B[a]P-induced CYP1A1 activity was observed at all concentrations tested. At 10 ppm, **hyperforin** exerted a strong decreasing effect. This is quite interesting, as all tested concentrations are below the plausible concentration in the intestinal tract (10 ppm to 100 ppm for hyperforin).

These results are consistent with the results obtained with reporter gene assays (see above) showing an inhibitory effet of both hypericin and hyperforin on the AhR activation by TCDD.

The antagonistic effect of **hypericin** and **hyperforin** on CYP1A1 induction by B[a]P may be considered as positive regarding detoxification of living organisms submitted to PAHs. St John's wort could have a beneficial effect for the consumer by limiting the activation of co-occurring procarcinogenic molecules. Schwarz et al. previously observed the inhibition of recombinant CYP1A1 enzymatic activity by hypericin and hyperforin (Schwarz et al., 2003). In their study, based on a different biological model (microsomal systems), they suggest that inhibition by **hyperforin** results from competition with the substrate B[a]P for its binding site on the CYP1A1 enzyme. On the other hand, **hypericin**, the other active ingredient, showed a noncompetitive inhibition of CYP1A1.

The inhibitory effect of the active compounds on CYP1A1 activity in presence of B[a]P may be put in relationship with the antagonist activity on the aryl hydrocarbon receptor (AhR), in human dioxin-sensitive cells (see above). In the scientific literature, B[a]P is known to bind with high specificity to the aryl hydrocarbon receptor (AhR), modifying the expression of CYP1A1. For example, it has been shown that B[a]P can significantly increase AhR and CYP1A1 expression in the human skin (Costa et al., 2010). Both induction and inhibition effects on CYP1A1 activity of the different active ingredients could occur through the AhR regulation pathway. Further investigations would be interesting.

The inhibitory effect of the active ingredients taken independently has been enlarged to whole crude St. John's wort preparations. Schwarz & al. observed that St. John's wort extracts (from 3 commercial products) have the capacity to inhibit the diolepoxidation activity of CYP1A1 (Schwarz et al., 2003). For the observed inhibition of CYP1A1, it would be difficult to determine which constituent(s) is responsible for the CYP1A1 inhibition. Hypericin and hyperforin were found the most potent inhibitors. Thus, they are most likely responsible for the inhibition effect of the whole extract. However, besides the inhibitory potency, in the case of such a complex

mixture of constituents, the relative abundance of each compound in the preparation would also have an impact on the contribution of a distinct compound to inhibition, and other constituents of St. John's wort may contribute as well (Schwarz et al., 2003). St. John's wort preparations are complex mixtures of a huge variety of compounds. The concentrations of the various constituents and their inhibitory potencies are different; therefore, it is difficult to single out the main inhibitor of CYP1A1 activity under *in vitro* conditions. The *in vivo* situation of CYP1A1 inhibition is even more complicated because pharmacokinetic properties (*e.g*., distribution, bioavailability, intracellular distribution, and clearance) must be taken into account (Schwarz et al., 2003).

The modulation of the CYP1A1 activity by the active ingredients hypericin and hyperforin has thus been observed in this study, and is also documented on other biological models. This modulation doesn't take part in the reactions leading to their anti-depressant activity, thus could induce unknown actions on human health.

2.5.4.6 Interactions with the cytochrome CYP3A4 on Caco-2 cells

In this study, the same cell line Caco-2 has been used. The objective was to assess the influence of the active ingredients of St John's wort on the activity of the CYP3A4 cytochrome.

No inhibitory effect on CYP3A4 activity by hypericin or hyperforin was observed (not shown). Regarding CYP3A4 induction, treatment of two weeks with hyperforin 1 ppm increased CYP3A4 activity, but was also cytotoxic, since the amount of protein at the end of the assay dramatically decreased (results not shown). The results of the study are therefore difficult to interprete.

2.5.5 In vitro studies of active ingredients from soy isoflavones

2.5.5.1 General toxicity of the active ingredients of soy isoflavones

Genistein, daidzein and glycitein are the three major isoflavones that contribute for 40% of the soy extract. Their standards were used to perform different *in vitro* assays of toxicity and inhibition of proliferation. Results are present in TABLE XVI.

TABLE XVI: General toxicity and inhibition of proliferation assays for genistein, daidzein and glycitein. IC50: concentration at which 50% of the growth was inhibited, LOEC: Lowest Observed Effect Concentration, LC50: Lethal Dose at which 50% of the cells are dead. /: not tested, -: no effect. For Caco-2 cells, results are issued from graphical interpretation and not from statistical interpretation.

Active ingredients (plausible concentration)	*Bacteria*		*Eucaryotic cells*			
	E. coli growth inhibition (IC 50)	*E.coli growth inhibition (LOEC)*	*Cytotoxicity in HepG2 cells (LC50)*	*Cytotoxicity in HepG2 cells (LOEC)*	*Caco 2 cells proliferation (MTS assay)*	*Cytotoxicity on Caco 2 cells (LDH assay)*
Genistein (6 - 92 ppm)	**21,9 ppm**	**6,3 ppm**	> 100 ppm	**25 ppm**	Concentration tested 0,01 -> 50 ppm **Inhibition from 10 ppm**	-
Daidzein (11 - 58 ppm)	> 100 ppm	> 100 ppm	> 100 ppm	> 100 ppm	Concentration tested 0,01 ppm ->50 ppm **Inhibition from 1 ppm**	-
Glycitein (10 - 33 ppm)	/	/	/	/	Concentration tested 0,01ppm ->25ppm **Slight inhibition from 10 ppm**	-

Daidzein and genistein both displayed low toxicity on *E.Coli*, human HepG2 cells, in the range of plausible intestinal concentrations. In Caco-2 cells, a dramatic decrease of cell viability was observed with 50 ppm genistein, which is in the range of realistic concentrations. A slight inhibition of the proliferation of Caco-2 cells has been observed for glycitein and daidzein. Genistein seems to have an antimicrobial effect (on *E.Coli*) and its first effects of cytotoxicity on human HepG2 cells occur in the range of plausible intestinal concentrations.

In the scientific literature, genistein was already found to inhibit cell proliferation, to induce apoptotic cell death and differentiation in cancer cells, to exert antioxidant effects and to modulate cell cycle progression. At the cellular level, genistein was found to induce apoptosis and differentiation in cancer cells, inhibit cell proliferation, modulate the cell cycle, exert antioxidant effects, inhibit angiogenesis, and suppress osteoclast and lymphocyte functions. For the authors, these activities make genistein a promising agent in the treatment of cancer (Polkowski & Mazurek, 2000).

In the literature, it can be found that daidzein significantly inhibited cell proliferation in a dose- and time-dependent manner and resulted in significant cell cycle arrest after 72 h of treatment at concentrations over 5 and 10 µM (performed on breast cancer cells) (Choi & Kim, 2008). Those results match ours on Caco-2 human colon adenocarcinoma cells, where cell proliferation is inhibited from 1 ppm (4 µM), but not the liver HepG2 cancer cells. These differences can be explained by the specificity of the cells. Nevertheless, as for the genistein, daidzein could exert its anticancer effects in human colon cells. The putative anticancer properties are not well supported by data and need further investigations to be put forward.

2.5.5.2 Effect of active ingredients of soy isoflavones on targeted stress genes

A battery of genetically modified *E.coli* bacteria was used to gain insight in the stress-related mode of action of genistein, daidzein and glycitein. Based on the toxicity data, appropriate concentration ranges were determined for the bacterial gene expression

assay. Four different modes of action classes were analyzed: oxidative damage, DNA damage, general cell lesions and membrane damage. Detailed bacterial strains are presented on TABLE XVII.

TABLE XVII: *E.coli* induced stress genes. In blue: significant induction, -: no induction

	E.Coli significant induced genes			
	oxidative damage	***membrane damage***	***cellular stress***	***DNA damage***
Active ingredients	*KatG, Zwf, Soi28, Nfo, MerR*	*MicF, OsmY*	*UspA, ClpB*	*RecA, UmuDC, Ada, DinD, SfiA*
Genistein	**MerR, Soi28, Nfo**	-	-	**DinD**
Daidzein	-	-	-	-
Glycitein	-	-	-	-

In the literature, numerous *in vitro* eukaryotic cell culture studies have reported genistein to be clastogenic (chromosome breaking), DNA damaging and even mutagenic, in contrast to *in vivo* studies that generally have not showed genotoxicity results. Genistein would act to both induce oxidative DNA damage and to act as an antioxidant to potentially prevent such damage. The concentrations at which these opposing effects occur are difficult to reconcile (Klein & King, 2007).

2.5.5.3 Dioxin-like activity of active ingredients of soy isoflavones

Agonistic assays

Dioxin sensitive cells were exposed to each isoflavone (in a range of concentrations from 2.5 to 80 μM during 6h and 24h to assess the dioxin-like activity (agonistic activity)). The results are shown in TABLE XVIII.

TABLE XVIII: Agonistic activities of isoflavones in dioxin-sensitive cells exposed during 6h or 24h to active ingredients. rAhH: rat Ah receptor; hAhR: human Ah receptor; -: no activity; +: weak activity (<25% of the TCDD maximal response); ++: medium activity (25% <effect <75% of the TCDD maximal response); Number in brackets: minimal concentration showing an activation (corresponding to 15% of the maximal response obtained with TCDD).

	ACTIVATION			
Active ingredients (PC in intestine)	***rAhR (6h cell exposure)***	***rAhR (24h cell exposure)***	***hAhR (6h cell exposure)***	***hAhR (24h cell exposure)***
Genistein (6 - 92 ppm)	++ (11 ppm)	-	-	-
Daidzein (11 - 58 ppm)	++ (5 ppm)	+ (10 ppm)	-	-
Glycitein (10 - 33 ppm)	++ (10 ppm)	-	-	-

Isoflavones exerted a "medium" rAhR agonistic activity when cells are exposed during 6h, but no more after 24h (except a "residual" weak activity for daidzein). In contrast, no activity was measured in human hepatoma cells. These

results suggest that the isoflavones are able to induce the AhR pathway in rat but in a transient way. They are probably metabolized in these liver cells and are not present anymore after 24h. The discrepancy between rat and human cells could result from the difference of cell sensitivity towards dioxins.

Antagonistic assays

"Antagonistic assays" on rat hepatoma cells revealed that some "isoflavone + TCDD" mixtures exert an additive or synergistic activity in rat cells (TABLE XIX). Compared to exposure to isoflavone alone (Figure 14), a synergy was observed between "daidzein and TCDD" (6h). An equivalent synergy was also observed between "genistein and TCDD" (6h).

TABLE XIX: antagonistic activities of isoflavones in dioxin-sensitive cells co-exposed (during 6h or 24h) to TCDD and each active ingredients. rAhH: rat Ah receptor; hAhR: human Ah receptor; -: no activity; +: antagonistic activity (response < 75% of the TCDD maximal response); synergy : response >100 % of the TCDD maximal response. Number in brackets: minimal concentration showing an inhibition of the AhR (corresponding to 75% of the maximal response obtained with TCDD alone).

	INHIBITION			
Active ingredients (PC in intestine)	***rAhR (6h cell exposure)***	***rAhR (24h cell exposure)***	***hAhR (6h cell exposure)***	***hAhR (24h cell exposure)***
Genistein (6 - 92 ppm)	synergy "TCDD-genistein" (5 ppm)		+ (22 ppm)	+ (11 ppm)
Daidzein (11 - 58 ppm)	synergy "TCDD-daidzein" (5 ppm)	synergy "TCDD-daidzein" (10 ppm)	-	-
Glycitein (10 - 33 ppm)	-	-	-	-

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 10nM of TCDD (antagonistic activity of genistein).

This could be a positive result for human health. None of the major active ingredients has a dioxin-like activity on human hepatoma cells HepG2-XRE nor do they synergize with the dioxin TCDD. Even better, genistein seems to antagonize TCDD induction. However, interference with AhR activity could also be deleterious for the normal detoxification functions of its target genes, therefore any collateral effect, such as the one observed here, has to be considered with precaution.

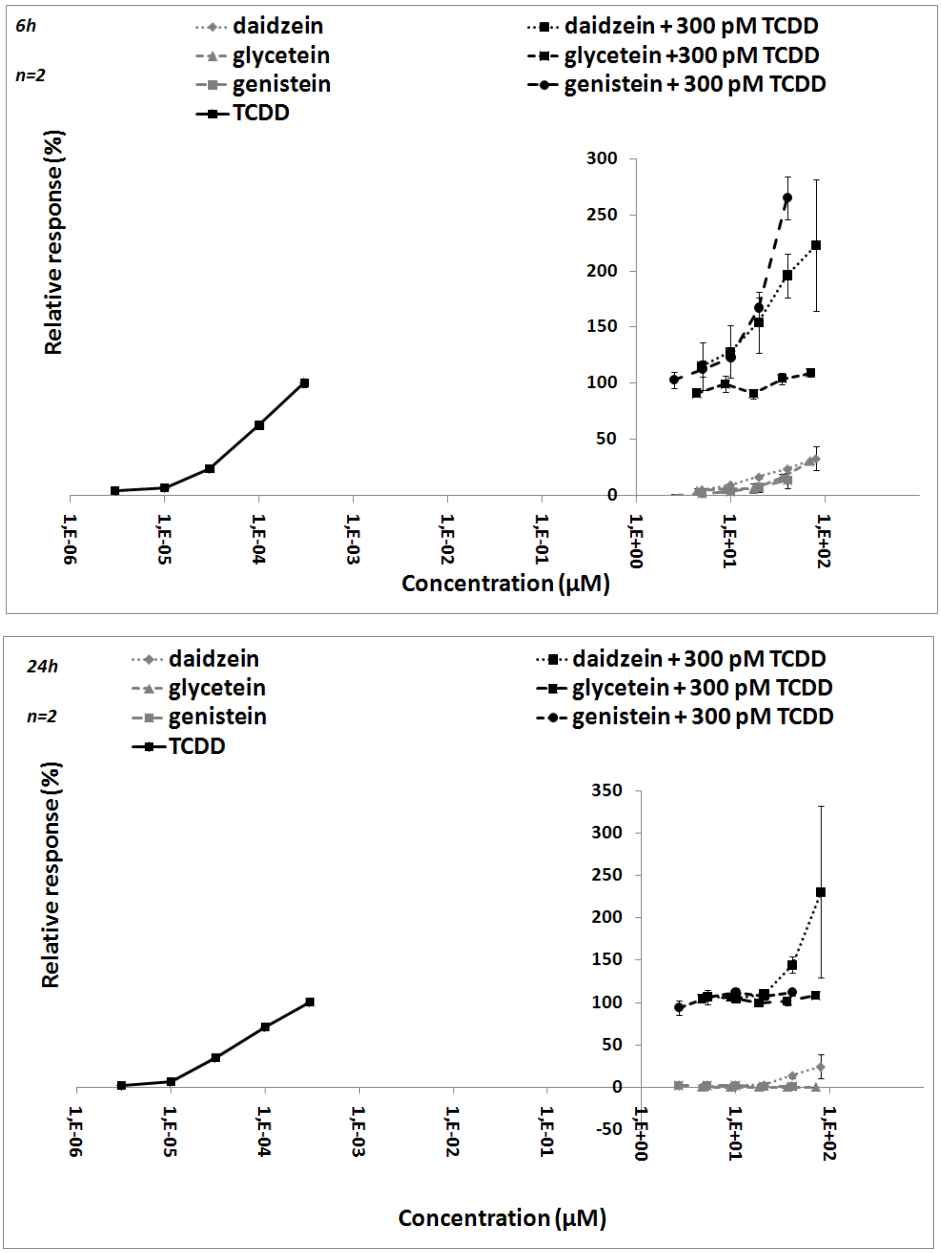


Figure 14: Comparison of mixtures "isoflavone + TCDD" and agonistic activities of isoflavones in rat hepatoma cells H4IIE-XRE. Above: exposure time of 6h. Below: exposure time of 24h.

2.5.5.4 Hormonal-like activities of active ingredients of soy isoflavones

Agonistic assays

Results of agonistic assays are shown in TABLE XX.

TABLE XX: agonistic activities of isoflavones in hormonal sensitive cells.
-: no activity; +++: strong activity (response > 75% of the reference ligand maximal response). Number in brackets: minimal concentration showing an activation of the steroid receptor (corresponding to 15% of the maximal response obtained with the reference ligand).

	ACTIVATION			
Active ingredients (PC in intestine)	***human AR***	***human PR***	***human GR***	***human ER***
Genistein (6 - 92 ppm)	-	-	-	+++ (0,03 ppm)
Daidzein (11 - 58 ppm)	-	-	-	+++ (0,1 ppm)
Glycitein (10 - 33 ppm)	-	-	-	+++ (2,6 ppm)

We concluded that isoflavones display estradiol-like activities and thus should be classified as phytoestrogens, in agreement with the literature. The fact that soy isoflavones have an estrogen-like activity is the main argument for treating menopausal symptoms.

Antagonistic assays

The results of two independent experiments were compared to the maximal response obtained with the reference ligand of each steroid receptor (hAR: dehydrotestosterone, hPR: progesterone, hGR: dexamethasome and hER: 17beta-estradiol) and are shown in TABLE XXI.

TABLE XXI: antagonistic activities of isoflavones in hormonal-sensitive cells co-exposed to the receptor reference ligand (see above) and each active ingredient. -: no activity; +: antagonistic activity (response < 75% of the reference ligand maximal response); synergy : response > 100% of the reference ligand maximal response. Number in brackets: minimal concentration showing an inhibition of the steroid receptor (corresponding to 75% of the maximal response obtained with the reference ligand).

	INHIBITION			
Active ingredients (PC in intestine)	***human AR***	***human PR***	***human GR***	***human ER***
Genistein (6 - 92 ppm)	-	+ (5,4 ppm)	+ (10,8 ppm)	synergy "17β-estradiol-Genistein" (0,03 ppm)
Daidzein (11 - 58 ppm)	synergy "5αDHT-Daidzein" (20,4 ppm)	+ (10,2 ppm)	+ (20,4 ppm)	synergy "17β-estradiol-Daidzein" (0,05 ppm)
Glycitein (10 - 33 ppm)	-	+ (11,4 ppm)	+ (19,9 ppm)	synergy "17β-estradiol-Glycetein" (1,3 ppm)

Results showed that the mixture "daidzein + dehydrotestosterone" and each mixture of "isoflavone + estradiol" produced a synergistic response (details not shown).

For each isoflavone, an inhibition of the progestagen and the glucocorticoid pathways occurred. Progestagen controls the maintenance of pregnancy (a disruption can be responsible of abortion or early birth), bone metabolism, neuronal development, and reproductive system development. Inhibition of hPR by isoflavones could thus probably is a matter of concern. The glucocorticoid pathway (dexamethasone) controls glucose metabolism (equilibrium of the glucose concentration in blood) and inflammation. A chronic exposure to an excessive concentration of glucocorticoids is linked to obesity, type 2 diabetes and inflammation diseases.

In estrogen-sensitive cells, the response was more than 100 % of the estradiol maximal response when each isoflavone was added to the cells (see TABLE XXI). The results obtained with mixtures with the reference ligand and the analysis of the different curves led us to conclude to a synergy between the three isoflavones and estradiol (details not shown). Considerations about the pro and cons of phytoestrogens are described in the chapter 2.6.2.

2.5.5.5 Interactions of active ingredients of soy isoflavones with the cytochrome CYP1A1 on Caco-2 cells

Caco-2 cells have been exposed during 6h or 24h to soy isoflavones (genistein, daidzein or glycitein), in presence or not of B[a]P, a well-known contaminant inducing CYP1A1 activity.

Results are presented in TABLE XXII and Figure 15.

TABLE XXII: Impact of pure compounds as standards on CYP1A1 activity in presence or absence of B[a]P, in 16-day post-confluent Caco-2 cells. -: no effect. For Caco-2 cells, results are issued from graphical interpretation and not from statistical interpretation.

	Concentrations tested (ppm)	***Impact on CYP1A1 activity*** *6h and 24h exposure on 16-day post-confluent cells*	***Impact on B[a]P- induced CYP1A1 activity*** *6h exposure on 16-day post-confluent cells*
Genistein	1; 12,5 and 25	-	Strong inhibition from 12,5 ppm
Daidzein	1; 12,5 and 25	-	Inhibition from 12,5 ppm
Glycetein	1; 12,5 and 25	-	Inhibition from 12,5 ppm

No induction of CYP1A1 activity in Caco-2 cells has been measured after 6h and 24h of exposure to genistein, daidzein or glycitein for the different concentrations tested. However, although not observed here, Sergent & al. observed previously that genistein presents a dose-dependant inducing effect (from 2.5 ppm) on CYP1A1

activity (Sergent, et al., 2009). Because genistein does not affect CYP1A1 mRNA expression, they suggest that it is not an aryl hydrocarbon receptor (AhR)-ligand in intestinal cells, but rather acts at a post-transcriptional level. This can also be related to the absence of agonist effect on AhR on human hepatoma cells (see chapter 2.5.5.3).

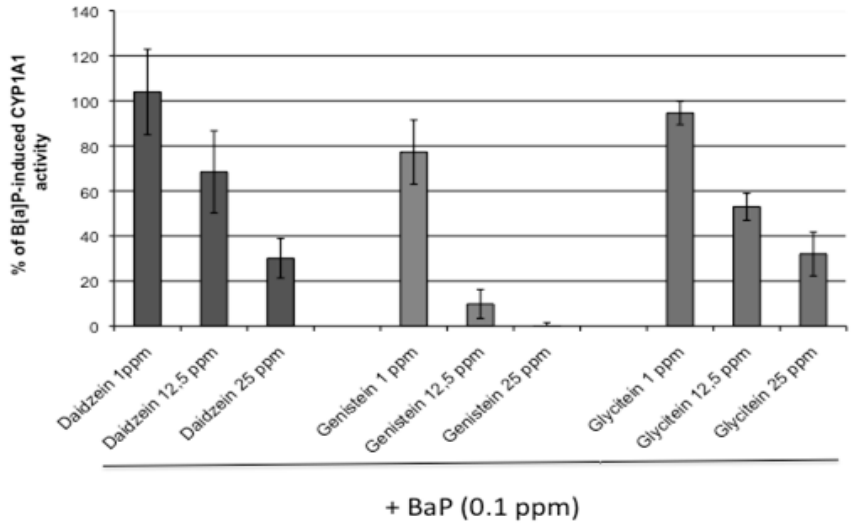


Figure 15: Decreasing effect of different concentrations of soy isoflavones on B[a]P-induced CYP1A1 activity in Caco-2 cells. Sixteen-day post-confluent cells seeded in 48-well plates (4.10^4 cells/cm^2) were exposed to 0.1 ppm of B[a]P alone or simultaneously with 1, 12.5 or 25 ppm of daidzein, genistein or glycitein for 6h. CYP1A1 activity was assessed through the measurement of the fluorescence produced by the conversion of 5 μM 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin produced/min/mg protein and presented in % of B[a]P-induced CYP1A1 activity. Values are means ± S.E.M. of three independent experiments performed in triplicates (n=9).

In the antagonistic assays with B[a]P, co-exposure with any of the three polyphenols caused a decrease of B[a]P-induced CYP1A1 activity at all concentrations tested. At 12,5 ppm, the three isoflavones showed a marked antagonistic effect.

Genistein and daidzein have already been reported to inhibit CYP1A1 activity or gene expression in different models (Gradin et al., 1994; Helsby et al., 1998; Kim et al., 2000; Hukkanen et al., 2000; Shon et al; 2006).

The same conclusions can be given as for the active ingredients of St John's wort (see chapter 2.5.4.5): even if, in a way, they modulate the CYP1A1 activity in a potentially positive direction, these compounds are not consumed in food supplements for that purpose. Any collateral action of the active compounds should be completely known and controlled.

2.5.5.6 Interactions with the cytochrome CYP3A4 on Caco-2 cells

No *in vitro* tests were performed in this project to study the interaction of genistein, daidzein and glycitein with the cytochrome CYP3A4. Nevertheless, it is interesting to notice that this has been done before with some flavonols, among which the genistein. Genistein was detected as significant inhibitor of the 1,25-vitamin D3-induced CYP3A4 activity (Sergent, et al., 2009). *In vivo*, these effects could result in reduced activation of procarcinogens, such as aflatoxin B1, and/or in drug bioavailability modulation. Authors underline the importance of intestinal studies to assess food safety and health risks linked to the ingestion of flavonoid-enriched supplements or functional foods.

2.5.6 In vitro studies of active ingredient and extract from Ginkgo biloba

For *Ginkgo biloba*, pure active ingredients were tested separately and in mixtures as well as a *Ginkgo biloba* extract (GBE). The GBE used in the present study was provided by the U.S. National Institute of Standards and Technology (NIST, SRM 3247) (www.nist.gov/srm).

TABLE XXIII: Certified concentration values (mg/g) for active ingredients in the standardized *Ginkgo biloba* extract (GBE, SRM 3247), as provided by the supplier (NIST), as well as calculated daily ingested dose (mg/day) and maximal realistic intestinal concentration ($C_{intestinal}$; μg/ml) of the different active ingredients, taking into account that 100 mg of extract are ingested per day and are diluted in 1l of gastro-intestinal fluid (100 μg/ml).

National Institute of Standards and Technology NIST
...working with industry to foster innovation, trade, security and jobs

Active ingredients	**Certified concentrations in the extract (SRM 3247)**	**Suggested working dose: 100 mg extract/day**	
	Mass fraction (mg/g dry weight)	mg/day	$C_{intestinal}$ (μg/ml)
Gingkolide A	**11.6** ± 1.7	1.16	**1.2**
Gingkolide B	**5.92** ± 0.45	0.592	**0.6**
Gingkolide C	**12.4** ± 1.4	1.24	**1.2**
Gingkolide J	**3.9** ± 1.5	0.39	**0.4**
Bilobalide	**28.5** ± 2.1	2.85	**2.8**
Quercetin	**45.1** ± 4.6	4.51	**4.5**
Kaempferol	**40.8** ± 3	4.08	**4.0**
Isorhamnetin	**10.8** ± 1.3	1.08	**1.0**

Mix terpenes
Mix flavonols
Whole mix

Individual active ingredients have been tested at the concentration occurring in 100 μg of ginkgo NIST standardized extract/ml, which is considered as a plausible intestinal concentration. These intestinal concentrations are given in TABLE XXII as well as the certified concentration values in the NIST extract for each active ingredient considered in the present work and furnished by the supplier.

The active ingredients composition and concentration of the three different mixtures tested with our *in vitro* assays are indicated in TABLE XXIII as well ("mix terpenes", "mix flavonols", and "whole mix" including the 8 compounds).

2.5.6.1 General toxicity of the active ingredients of *Ginkgo biloba*

The results are presented in TABLE XXIV.

Kaempferol, one of the three flavonols, is the most potent compound of the different active ingredients that were analyzed both with the bacterial and human *in vitro* assays. Improved risk assessment for kaempferol might be needed since it occurs in high concentrations and is the most potent compound.

TABLE XXIV: General toxicity and inhibition of proliferation assays for the *Ginkgo biloba* active ingredients and mix of active ingredients. IC50: concentration at which 50% of the growth was inhibited, LOEC: Lowest Observed Effect Concentration, /: not tested; EDI: Estimated Daily Intake. For Caco-2 cells, results are issued from graphical interpretation and not from statistical interpretation.- : no effect.

Active ingredients (plausible concentration)	*Bacteria*		*Eucaryotic cells*		
	E. coli growth inhibition (IC 50)	*E.coli growth inhibition (LOEC)*	*Cytotoxicity in HepG2 cells (LOEC)*	*Caco 2 cells proliferation (MTS assay)*	*Cytotoxicity on Caco 2 cells (LDH assay)*
Ginkgolide A (0,1 - 7,8 ppm)	> 50 ppm	> 50 ppm	> 50 ppm	-	-
Ginkgolide B (0,1 - 10,4 ppm)	> 50 ppm	> 50 ppm	> 50 ppm	-	-
Ginkgolide C (0,4 - 3,1 ppm)	> 50 ppm	> 50 ppm	> 50 ppm	-	-
Ginkgolide J (0,2 - 2,9 ppm)	> 50 ppm	> 50 ppm	12,5 ppm	Slight inhibition at 25 ppm	-
bilobalide (0,6 - 13,6 ppm)	> 50 ppm	> 50 ppm	6,3 ppm	-	-
Isorhamnetin (1,3 - 19,2 ppm)	> 50 ppm	> 50 ppm	6,3 ppm	Inhibition from 10 ppm	-
kaempferol (1,3 - 22,4 ppm)	12,5 ppm	1,6 ppm	6,3 ppm	Inhibition from 10 ppm	-
Quercetin (1,3 - 81,4 ppm)	/	/	/	Slight inhibition at 25 ppm	-
mix 8 compounds stock diluted 200 =EDI	0,5 times EDI	0,03 times EDI	/	/	-
flavonols	0,5 times EDI	0,03 times EDI	/	/	-
mix terpènes	-	-	/	/	-
standard extract 696 µg/ml = 1,74 times EDI	-	0,10 times EDI	/	/	-

The ″Mix 8 compounds″ induce a growth inhibition on bacterial cells when 50% of the estimated daily intake (EDI) is reached. Because the investigated cells (*E.coli*) are the majority present in the digestive track, this could provoke a rupture in the bacterial equilibrium when high doses of *Ginkgo biloba* are taken. Nevertheless, the standard extract doesn't induce that antimicrobial effect. Further investigations on food supplements could be interesting for a better understanding.

2.5.6.2 Effect of active ingredients of *Ginkgo biloba* on targeted stress genes

The results are shown in TABLE XV.

As in the previous chapter, kaempferol induces a significant number of genes, related to various kinds of stresses. A negative impact of kaempferol on the intestinal and hepatic cells may be possible. The terpen ginkgolide A presented low impact compared to kaempferol. Improved risk assessment for kaempferol might be needed since it occurs in high concentrations and is the most potent compound.

TABLE XXV : *E.coli* induced stress genes. In blue: significant induction, -: no induction.

	bacterial E.Coli significant induced genes			
Active ingredients	***oxidative damage*** *KatG, Zwf, Soi28, Nfo, MerR*	***membrane damage*** *MicF, OsmY*	***cellular stress*** *UspA, ClpB*	***DNA damage*** *RecA, UmuDC, Ada, DinD, SfiA*
Ginkgolide A (0,1 - 7,8 ppm)	-	-	-	DinD
Ginkgolide B (0,1 - 10,4 ppm)	-	-	-	-
Ginkgolide C (0,4 - 3,1 ppm)	-	-	-	-
Ginkgolide J (0,2 - 2,9 ppm)	-	-	-	-
bilobalide (0,6 - 13,6 ppm)	-	-	-	-
Isorhamnetin (1,3 - 19,2 ppm)	-	-	-	-
kaempferol (1,3 - 22,4 ppm)	Zwf	-	UspA, ClpB	UmuDC
Quercetin (1,3 - 81,4 ppm)	/	/	/	/
mix 8 compounds stock diluted 200 =EDI	-	-	ClpB	-
flavonols	-	-	ClpB	RecA
mix terpènes	-	-	-	-
standard extract 696 µg/ml = 1,74 times E	-	-	-	-

Nevertheless, it is important to notice that the beneficial effects of ginkgo leaves are the antioxidant properties. Antioxidants are substances that scavenge free radicals, which are damaging because they alter cell membranes, tamper with DNA, and even cause cell death. Free radicals occur naturally in the body, but environmental toxins (including ultraviolet light, radiation, cigarette smoking, and air pollution) can also increase the number of these damaging particles. Free radicals are believed to contribute to a number of health problems including heart disease and cancer as well as Alzheimer's disease and other forms of dementia. Antioxidants such as those found in ginkgo can neutralize free radicals and may reduce or even help prevent some of the damage they cause. As the used *in vitro* assay can only assess the presence of antioxidant inducing compounds, an interesting experiment would be to perform a mixture exposure to a typical oxidative stress inducing compound (ex. paraquat) and *Ginkgo biloba* to confirm the antioxidant properties.

2.5.6.3 Dioxin-like activity of active ingredients of *Ginkgo biloba*

Agonistic assays

Table XXVI presents the results after 6 hours of cell exposure only, because no dioxin-like activity was observed after 24 hour. Only ginkgolide J exerted a medium agonistic activity in rat hepatoma cells. Bilobalide and quercetin had weak

agonistic activity when exposed to the human hepatoma cells. These results suggest that the dioxin-like activities of some GBAI are rather weak and transient in these cells.

TABLE XXVI: agonistic activities of *Ginkgo biloba* active ingredients and *Ginkgo biloba* extract in dioxin-sensitive cells. -: no activity; +: weak activity (response <25% of the TCDD maximal response); ++: medium activity (25% <response <75% of the TCDD maximal response). Number in brackets: minimal concentration (ppm) showing an activation (corresponding to 15% of the TCDD maximal response) of the Ah receptor.

	ACTIVATION	
Active ingredients	***AhR in rat hepatoma cells***	***AhR in human hepatoma cells***
	ppm	***ppm***
Ginkgolide A (0,1 - 7,8 ppm)	-	-
Ginkgolide B (0,1 - 10,4 ppm)	-	-
Ginkgolide C (0,4 - 3,1 ppm)	-	-
Ginkgolide J (0,2 - 2,9 ppm)	++ (10,0)	-
bilobalide (0,6 - 13,6 ppm)	-	+ (9,8)
Isorhamnetin (1,3 - 19,2 ppm)	-	-
kaempferol (1,3 - 22,4 ppm)	-	-
Quercetin (1,3 - 81,4 ppm)	-	+ (6,8)
Mix 8 compounds	++ (6,3)	+ (12,6)
Mix terpens	-	-
Mix flavonols	-	+ (7,6)
Gingko biloba extract	++ (25,4)	++ (6,4)

The mix of the flavonols has a weak dioxin-like activity when exposed to the human hepatoma cells. This effect is mainly due to quercetin, however a synergistic activity of the other flavonols in the mixture might also contribute. When mixed to the other terpens, the dioxin-like effect of ginkgolide J seems negligible because of its low concentration in the mix. For the mix of the eight compounds, a weaker dioxin-like activity is observed compared to the one observed using the "Mix flavonols", when exposed to the human hepatoma cells. These results suggest an inhibition of the flavonol effect by the terpens.

The "GB extract" from an international reference displays a stronger dioxin-like activity than the mix 8 compounds, suggesting an additive effect of quercetin and bilobalide.

Our results are the demonstration that there are complex interactions between the eight active compounds, and that other components of the *Ginkgo biloba* extracts can also interfere with the activity of the food supplement. Considering the matrix (the caps, the pill, compacting ingredients,…), the activity can be far from the expected one.

Antagonistic assays

Results are presented in TABLE XXVII.

TABLE XXVII: antagonistic activities of *Ginkgo biloba* active ingredients and *Ginkgo biloba* extract in dioxin-sensitive cells co-exposed to TCDD and the tested compounds. -: no activity; +: antagonistic activity (response < 75% of the TCDD half maximal response); Number in brackets: minimal concentration showing an inhibition (corresponding to 75% of the TCDD half maximal response) of the Ah receptor. /: not tested.

	INHIBITION			
Active ingredients (PC in intestine)	***rAhR (6h cell exposure)***	***rAhR (24h cell exposure)***	***hAhR (6h cell exposure)***	***hAhR (24h cell exposure)***
	ppm			
Ginkgolide A (0,1 - 7,8 ppm)	-	-	-	-
Ginkgolide B (0,1 - 10,4 ppm)	-	-	-	-
Ginkgolide C (0,4 - 3,1 ppm)	-	-	-	-
Ginkgolide J (0,2 - 2,9 ppm)	-	-	-	-
bilobalide (0,6 - 13,6 ppm)	-	-	+ (19,6)	-
Isorhamnetin (1,3 - 19,2 ppm)	+ (10,1)	-	+ (1,3)	+ (6,3)
kaempferol (1,3 - 22,4 ppm)	-	-	+ (1,1)	+ (5,1)
Quercetin (1,3 - 81,4 ppm)	+ (13,5)	+ (13,5)	+ (1,7)	+ (6,3)
Mix 8 compounds	-	/	+ (1,6)	/
Mix terpens	-	/	-	/
Mix flavonols	+ (7,6)	/	+ (1,0)	/
Gingko biloba extract	synergy "TCDD-GB extract (3,2)	/	+ (12,7)	/

The flavonols independently seem to have an antagonistic activity. Because of their weak and transient dioxin-like activity, this could be considered as positive. Nevertheless, as for the agonistic assays, the variability of the results suggests the importance of the interactions between the different compounds of the *Ginkgo biloba*.

For example, the "GB extract" shows an antagonistic activity when exposed to the human hepatoma cells. The effect is weaker than that obtained with the "Mix 8 compounds", despite the fact that the exposed concentrations are similar. We can conclude of an inhibiting effect of the GB extract. On the contrary, in rat hepatoma cells, agonistic activity was observed: the results showed that mixture of "GB extract

+ TCDD" produced a relative response higher than 100%. We concluded to a synergy.

Those results, even if obtained from standard active ingredients (independently and mixed), suggest that the *Ginkgo biloba* food supplements may modulate the response to dioxin compounds. This action is not part of the reactions leading to their therapeutic activities, thus could induce unknown impacts on human health.

2.5.6.4 Hormonal-like activity of active ingredients of *Ginkgo biloba*

Agonistic assays

TABLE XXVIII: agonistic activities of *Ginkgo biloba* active ingredients and *Ginkgo biloba* extract in hormonal-sensitive cells. -: no activity; ++: medium activity (25% < response <75%) of the reference ligand maximal response; +++: strong activity (response > 75% of the reference ligand maximal response); Number in brackets: minimal concentration (ppm) showing an activation (corresponding to 15% of the maximal response obtained with the ligand of reference). /: not tested.

	ACTIVATION			
Active ingredients (PC in intestine)	***human AR***	***human PR***	***human GR***	***human ER***
		ppm		
Ginkgolide A (0,1 - 7,8 ppm)	-	-	-	-
Ginkgolide B (0,1 - 10,4 ppm)	-	-	-	-
Ginkgolide C (0,4 - 3,1 ppm)	-	-	-	-
Ginkgolide J (0,2 - 2,9 ppm)	-	-	-	-
bilobalide (0,6 - 13,6 ppm)	-	-	-	-
Isorhamnetin (1,3 - 19,2 ppm)	-	-	-	+++ (2,5)
kaempferol (1,3 - 22,4 ppm)	-	-	-	+++ (0,6)
Quercetin (1,3 - 81,4 ppm)	-	-	-	++ (6,8)
Mix 8 compounds	/	/	/	+++ (6,3)
Mix terpens	/	/	/	-
Mix flavonols	/	/	/	+++ (3,8)
Gingko biloba extract	/	/	/	++ (25,4)

Results (TABLE XXVIII) showed that only flavonoids exerted estrogen-like activities. We concluded that flavonols, as observed for isoflavones (soy), have estradiol-like activities and thus could be classified as phytoestrogens..

The results obtained with the mixtures "Mix flavonols" and "Mix 8 compounds" indicate a synergy of the flavonols. In "Mix flavonols" and "Mix 8 compounds", the maximum exposed concentrations of flavonols are lower but the agonistic effect is very strong. For "GB extract", the effect is weaker than that obtained with the "Mix flavonols" and "Mix 8 compounds", despite the fact that the exposed concentrations are higher. We thus conclude of an inhibiting effect from the other compounds of the GB extract, which has still an oestrogen-like activity.

Antagonistic assays

Results are presented in TABLE XXIX.

The group of flavonoids (kaempferol, quercetin and isorhamnetin) exerted antagonistic activities in progestagen-sensitive cells TM-Luc. Kaempferol and quercetin exerted activities in androgen-sensitive cells. On the other hand, only kaempferol exerted anti-glucocorticoid activity and a synergic activity with estradiol in estrogen-sensitive cells.

TABLE XXIX: antagonistic activities of *Ginkgo biloba* active ingredients and *Ginkgo biloba* extract in hormonal-sensitive cells. -: no activity; +: antagonistic activity (response < 75% of the TCDD maximal response); /: not tested. *: visual cytotoxicity observed by microscopic examination of the cells.

	INHIBITION			
Active ingredients (PC in intestine)	***human AR***	***human PR***	***human GR***	***human ER***
			ppm	
Ginkgolide A (0,1 - 7,8 ppm)	-	-	-	-
Ginkgolide B (0,1 - 10,4 ppm)	-	-	-	-
Ginkgolide C (0,4 - 3,1 ppm)	-	-	-	-
Ginkgolide J (0,2 - 2,9 ppm)	-	-	-	-
bilobalide (0,6 - 13,6 ppm)	-	-	-	-
Isorhamnetin (1,3 - 19,2 ppm)	-	+ (2,5)	-	-
kaempferol (1,3 - 22,4 ppm)	+ (2,5)	+ (2,6)	+ (1,4)	Synergy "E2-kaempferol" (1,2 ppm)
Quercetin (1,3 - 81,4 ppm)	+ (41,3)	+ (6,8)	*	-
Mix 8 compounds	/	/	/	synergy "17b-estradiol-Mix 8" (0,8 ppm)
Mix terpens	/	/	/	-
Mix flavonols	/	/	/	synergy "17b-estradiol-Mix flavonols" (0,5 ppm)
Gingko biloba extract	/	/	/	**+ (25,4 ppm)**

The "GB extract" showed antagonistic activity when exposed to the estradiol-sensitive cells, which is in opposition with the "Mix flavonols" and "Mix 8 compounds". We can conclude of an inhibiting effect from the GB extract.

Because of the differences between the pure active ingredients, the different mix and the standard extract, it is predictable that each food supplement, with its own recipe, may have a specific action on hormonal sensitive cells. Further investigations should be performed.

2.5.6.5 Interactions of active ingredients of *Ginkgo biloba* with the cytochrome CYP1A1 on Caco-2 cells

In this report, Caco-2 cells have been exposed during 6h or 24h first, to the NIST reference standardized extract of *Ginkgo biloba* and to the three commercial food supplements, then, to individual active ingredients of *Ginkgo biloba*, and finally to different mixtures of active ingredients, in order to assess CYP1A1 activity, in presence or not of B[a]P, a well-known contaminant inducing CYP1A1 activity.

The results are presented in TABLE XXX and Figure 16 to Figure 19. Results observed with commercial food supplements of G*inkgo biloba* (GBE1, 2 or 3) are hardly interpretable, since the right concentration of flavonols in the extract is not known, due to the lack of commercial standards for glycosidic forms.

TABLE XXX: Effects on CYP1A1 activity after exposure of Caco-2 cells to major active compounds of *Ginkgo biloba*. GBE: *Ginkgo biloba* extracts from products presented as food supplements for the consumer.

	Impact on CYP1A1 activity	***Impact on B[a]P- induced CYP1A1 activity***
Ginkgolide A, B, C, J	-	-
(-)-Bilobalide	-	-
Isorhamnetin	-	Decreasing effect
Kaempferol	Slight induction (24h)	Decreasing effect
Quercetin.$2H_2O$	Slight induction (24h)	Decreasing effect
Mix 8 compounds	Slight induction	Decreasing effect
Mix terpens	-	-
Mix flavonols	Slight induction	Decreasing effect
GB standard extract	Induction	Slight decreasing effect
GBE1	Induction	Decreasing effect
GBE2	Induction	Decreasing effect
GBE3	Induction	Decreasing effect

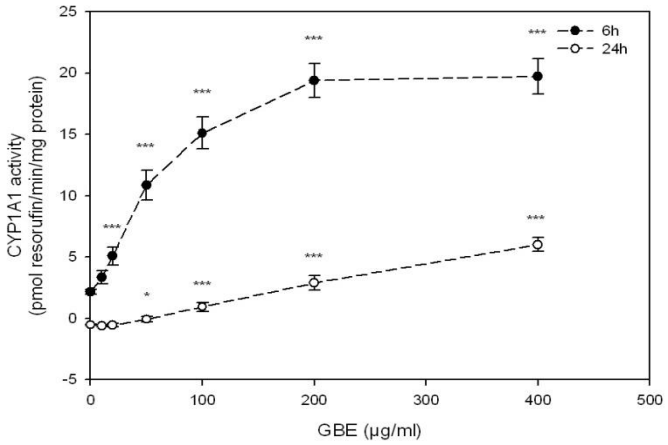


Figure 16: Induction of CYP1A1 activity in Caco-2 cells after 6h or 24h exposure to different concentrations of *Ginkgo biloba* extract (GBE). Sixteen-day post-confluent cells seeded in 48-well plates (4.10^4 cells/cm^2) were exposed to 10, 20, 50, 100, 200 or 400 ppm of *Ginkgo biloba* extract or to vehicle control (0.5% DMSO) for 6h or 24h. CYP1A1 activity was assessed through the measurement of the fluorescence produced by the conversion of 5 µM 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin produced/min/mg protein. Values are means ± S.E.M. of four independent experiments performed in triplicates (n=12).* $p<0.05$, ** $p<0.01$, *** $p<0.005$ as compared to vehicle control.

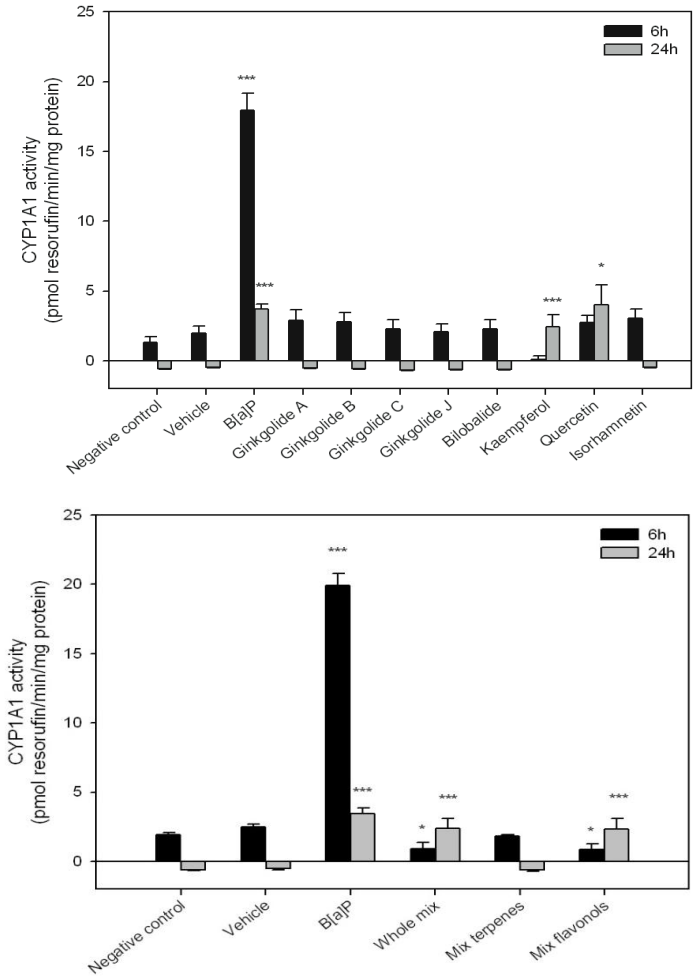


Figure 17: Induction of CYP1A1 activity in Caco-2 cells after 6h or 24h exposure to individual ingredients of *Ginkgo biloba* extract, alones (upper part) or in mixtures (lower part). Sixteen-day post-confluent cells seeded in 48-well plates (4.10^4 cells/cm^2) were exposed to each of the compounds listed in TABLE XXII, alone or in mixtures, at their assumed maximal realistic intestinal concentration or to controls for 6h or 24h. The negative control consisted in FBS-free culture medium alone, the vehicle control in 0.5% DMSO and the positive control in cells treated with BaP (0.1 μg/ml). CYP1A1 activity was assessed through the measurement of the fluorescence produced by the conversion of 5 μM 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin produced/min/mg protein. Values are means ± S.E.M. of four independent experiments performed in triplicates (n=12).* p<0.05, ** p<0.01, *** p<0.005 as compared to vehicle control.

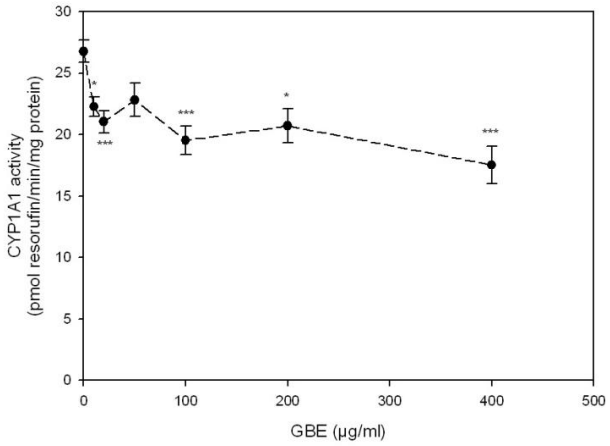


Figure 18: Decreasing effect of different concentrations of *Ginkgo biloba* extract (GBE) on B[a]P-induced CYP1A1 activity in Caco-2 cells. Sixteen-day post-confluent cells seeded in 48-well plates (4.10^4 cells/cm^2) were exposed to 0.1 ppm of B[a]P alone or simultaneously with 0, 10, 20, 50, 100, 200 or 400 ppm of *Ginkgo biloba* extract for 6h. CYP1A1 activity was assessed through the measurement of the fluorescence produced by the conversion of 5 µM 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin produced/min/mg protein. Values are means ± S.E.M. of four independent experiments performed in triplicates (n=12).).* p<0.05, ** p<0.01, *** p<0.005 as compared to B[a]P alone.

We observed a dose-dependent induction of CYP1A1 activity in Caco-2 cells, stronger after 6h exposure than 24h exposure to an extract of *Ginkgo biloba*. This induction after 6h exposure is not explained by the active ingredients tested separately or even in mixtures. Therefore, this strong effect may be due to other occurring compounds of the complex extract (flavonol glycosides, proanthocyanidins, carboxylic acids,…) (van Beek & Montoro, 2009). However, no literature data suggest such an induction of CYP1A1 activity by these other compounds. The slight induction of CYP1A1 activity observed after 24h exposure may be explained by quercetin and/or kaempferol.

In presence of B[a]P, the slight antagonistic effect on CYP1A1 activity observed with the extract of *Ginkgo biloba* may be explained by flavonols, which strongly decreased CYP1A1 activity in presence of B[a]P, when tested individually or even in mixtures, with or without terpens.

In the literature, inhibition has also been reported with isorhamnetin, kaempferol and quercetin at the level of CYP1A1 catalytic activity and/or gene expression. In the present work, terpens seem to be less active than flavonols towards CYP1A1.

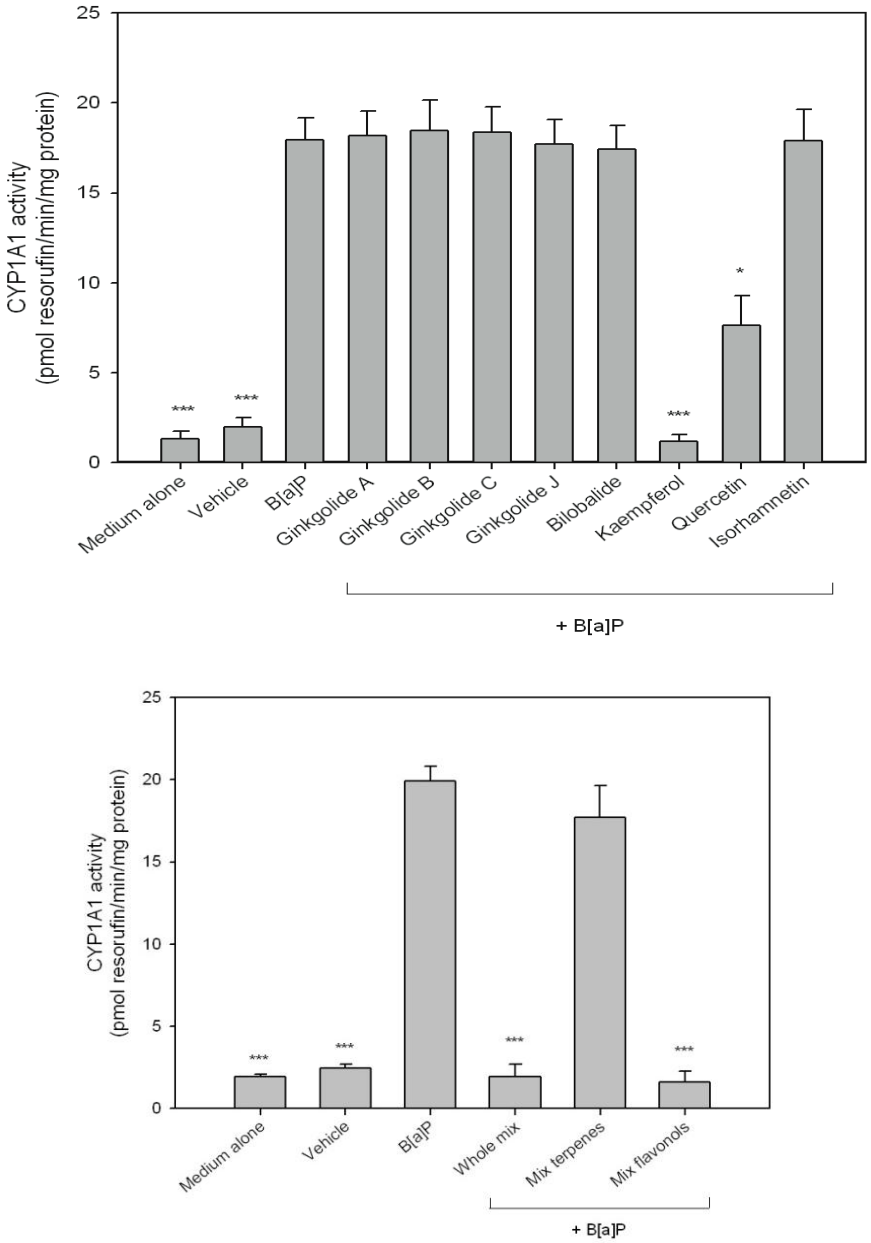


Figure 19: Decreasing effect of individual ingredients of *Ginkgo biloba* extract, alone (upper part) or in mixtures (lower part) on B[a]P-induced CYP1A1 activity in Caco-2 cells. Sixteen-day post-confluent cells seeded in 48-well plates (4.10^4 cells/cm^2) were exposed to 0.1 ppm of B[a]P alone or simultaneously with active ingredients listed in TABLE XXII alone or in mixtures, at their assumed maximal realistic intestinal concentration for 6h. The vehicle consisted in 0.6% DMSO. CYP1A1 activity was assessed through the measurement of the fluorescence produced by the conversion of 5 μM 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin produced/min/mg protein. Values are means ± S.E.M. of four independent experiments performed in triplicates (n=12).).* $p<0.05$, ** $p<0.01$, *** $p<0.005$ as compared to B[a]P alone.

A decreasing effect on CYP1A1 induction was observed after 6h exposure of Caco-2 cells simultaneously to B[a]P and GBE1, 2 or 3 at plausible intestinal concentration (not shown). Those results are similar to the ones above, but it is important to notice that the active ingredient concentrations in the raw extracts GBE1, 2 and 3 are unknown (100µg of each extract were used to perform the tests). As for the previous inhibitions of CYP1A1 activity by other active ingredients (from

soy and St John's wort) the inhibitory effect on the CYP1A1 activity could occur through the AhR regulation pathway.

Similar to St John's wort, we see that the active compounds of *Ginkgo biloba* products, independently and in mixtures, as well as extracts, 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 such as 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, extracts inducing CYP1A1 activity could participate to the conversion of pro-carcinogens to toxic DNA-binding metabolites, even if detoxification mainly occurred with most chemicals.

The modulation of the CYP1A1 activity is not part of the reactions leading to the therapeutic effects of the *Ginkgo biloba* food supplements thus could induce unknown collateral impacts on human health.

2.5.6.6 Interactions with the cytochrome CYP3A4 on Caco-2 cells

No *in vitro* tests were performed in this project to study the interaction of the *Ginkgo biloba* active ingredients with the cytochrome CYP3A4. Nevertheless, it is interesting to note that this has been done in the past.

Sergent & al. (2009) showed that quercetin inhibits the CYP3A4 constitutive activity, impairs the CYP3A4 induction by 1,25-vitD_3 and inhibits the 1,25-vitD_3-induced activity by direct enzyme interactions. As for grapefruit, the inhibition of the CYP3A4 activity may increase the plasma level of co-exposed drugs. The findings in humans are contradictory, and further studies are needed to elucidate the role of *Ginkgo biloba* in altered drug absorption due to CYP and P-gp inhibition. Three clinical studies excluded any effect of ginkgo on the CYP3A4 enzyme, but in two other studies ginkgo increased the plasma concentration of CYP3A4 probe drugs alprazolam and midazolam (Colalto, 2010).

2.5.7 Recapitulative results of *in vitro* assays

Recapitulative results obtained for the analysis of FS active ingredients using *in vitro* assays are summarized in the three following tables, as follow :

1. Summary of cytotoxicity and induction of stress genes
2. Summary of the effects on the AhR pathway and CYP1A1
3. Summary of the effects on the steroid receptors pathway

1. Cytotoxicity assays/stress genes induced

Food supplements	*Active ingredients (PC in intestine)*	*Bacteria*						*Eucaryotic cells*		
		E. coli growth inhibition (IC 50)	*E.coli growth inhibition (LOEC)*	*oxidative damage*	*membrane damage*	*cellular stress*	*DNA damage*	*Cytotoxicity in HepG2 cells (LOEC)*	*Cell proliferation inhibition in Caco-2 cells **	*Cytotoxicity (LDH release) in Caco-2 cells*
		ppm						*ppm*		
St John's wort	Hypericin (0,5 - 5 ppm)	38,25	12,5	-	-	-	-	12,5	12,5	-
	Hyperforin (10 - 100 ppm)	>10	> 10	-	-	-	-	**1,3**	**1,5**	-
Ginkgo biloba	Ginkgolide A (0,1 - 7,8 ppm)	**> 50**	**> 50**	-	-	-	**DinD**	> 50	-	-
	Ginkgolide B (0,1 - 10,4 ppm)	> 50	> 50	-	-	-	-	> 50	-	-
	Ginkgolide C (0,4 - 3,1 ppm)	> 50	> 50					> 50	-	-
	Ginkgolide J (0,2 - 2,9 ppm)	> 50	> 50	-	-	-	-	12,5	-	-
	bilobalide (0,6 - 13,6 ppm)	> 50	> 50	-	-	-	-	**6,3**	-	-
	Isorhamnetin (1,3 - 19,2 ppm)	> 50	> 50	-	-	-	-	**6,3**	**-**	-
	kaempferol (1,3 - 22,4 ppm)	**12,5**	**1,6**	**Zwf**	-	**UspA, ClpB**	**UmuDC**	**6,3**	**-**	-
	Quercetin (1,3 - 81,4 ppm)	/	/	/	/	/	/	/	**-**	-
	Mix 8 compounds	0,5 times EDI **	0,03 times EDI	-	-	ClpB	-	/	**/**	-
	Mix terpens	0,5 times EDI	0,03 times EDI	-	-	ClpB	RecA	/	**/**	-
	Mix flavonols	-	-	-	-	-	-	/	**/**	-
	Gingko biloba extract	-	0,10 times EDI	-	-	-	-	/	**/**	-
Soy isoflavones	Genistein (6 - 92 ppm)	**21,9**	**6,3**	**MerR, Soi28, Nfo**	-	-	**DinD**	**25**	**10**	-
	Daidzein (11 - 58 ppm)	> 100	> 100	-	-	-	-	> 100	**1**	-
	Glycitein (10 - 33 ppm)	/	/	-	-	-	-	/	**10**	-
Black Radish (tested concentrations)	L-sulforaphane (0,2 -> 12,5 µM)	183	3,2	KatG, MerR	OsmY	-	DinD	13	/	/
	DL-sulforaphane (1 -> 60 µM)	103	3,5	KatG, MerR	OsmY	ClpB	DinD	6	/	/

-: no effect
/: not tested
* These concentrations are LOEC (not based on statistical analysis but on visual interpretation)
** standard extract 696 µg/ml = 1,74 times EDI

2. AhR pathway/CYP1A1 activity

Food supplements	*Active ingredients (PC in intestine)*	*ACTIVATION*			*INHIBITION*		
		AhR in rat hepatoma cells	*AhR in human hepatoma cells*	*CYP1A1 in intestinal model Caco-2 cells ***	*AhR in rat hepatoma cells*	*AhR in human hepatoma cells*	*BaP induction of CYP1A1 activity in Caco-2 cells ***
				ppm			
St John's wort	Hypericin (0,5 - 5 ppm)	-	-	Slight induction at 10 ppm (6h and 24h exposure)	synergy "TCDD-hyperforin" (0,3)	**+ (1.3)**	Slight inhibition at all concentrations tested
	Hyperforin (10 - 100 ppm)	-	-	-	synergy "TCDD-hyperforin" (0.25)	**+ (0,5)**	Strong inhibition at 10 ppm
Ginkgo biloba	Ginkgolide A (0,1 - 7,8 ppm)	-	-	**- (1,2 ppm*)**	-	-	**- (1,2 ppm*)**
	Ginkgolide B (0,1 - 10,4 ppm)	-	-	**- (0,6 ppm*)**	-	-	**- (0,6 ppm*)**
	Ginkgolide C (0,4 - 3,1 ppm)	-	-	**- (1,2 ppm*)**	-	-	**- (1,2 ppm*)**
	Ginkgolide J (0,2 - 2,9 ppm)	**++ (10,0)**	-	**- (0,4 ppm*)**	-	-	**- (0,4 ppm*)**
	bilobalide (0,6 - 13,6 ppm)	-	**+ (9,8)**	**- (2,8 ppm*)**	-	**+ (19,6)**	**- (2,8 ppm*)**
	Isorhamnetin (1,3 - 19,2 ppm)	-	-	**- (1,0 ppm*)**	**+ (10,1)**	**+ (1,3)**	**- (1,0 ppm*)**
	kaempferol (1,3 - 22,4 ppm)	-	-	**Induction at 24h (4,0 ppm*)**	-	**+ (1,1)**	**Antagonism (4,0 ppm*)**
	Quercetin (1,3 - 81,4 ppm)	-	**+ (6,8)**	**Induction at 24h (4.5 ppm*)**	**+ (13,5)**	**+ (1,7)**	**Antagonism (4,5 ppm*)**
	Mix 8 compounds	**++ (6,3)**	**+ (12.6)**	**Induction (24h)**	-	**+ (1,6)**	**Strong antagonistic effect**
	Mix terpens	-	-	**-**	-	-	**-**
	Mix flavonols	-	**+ (7,6)**	**Induction (24h)**	**+ (8,0)**	**+ (1,0)**	**Strong antagonistic effect**
	Gingko biloba extract	**++ (25,4)**	**++ (6,4)**	**from 20 ppm (6h) and from 50 ppm (24h)**	synergy "TCDD-GB extract" (3,2)	**+ (12,7)**	**Slight antagonistic effect from 10 ppm**
Soy isoflavones	Genistein (6 - 92 ppm)	**++ (10,8)**	-	-	synergy "TCDD-genistein" (5,0)	**+ (10,8)**	**1,0**
	Daidzein (11 - 58 ppm)	**++ (5,0)**	-	-	synergy "TCDD-daidzein" (5,0)	-	**12,5**
	Glycitein (10 - 33 ppm)	**++ (10,0)**	-	-	-	-	**12,5**

PC: Plausible concentration
-: no effect
/: not tested
*** These concentrations are those occurring in the intestinal realistic concentration of 100ug GBE/ml (standardized GBE with certified concentrations by NIST = SRM3247)**
** These concentrations are not based on statistical analysis but on visual interpretation, except for ginkgo biloba
Number in brackets: minimal concentration showing an activation (corresponding to 15% of the TCDD maximal response) or an inhibition (corresponding to 75% of the TCDD maximal response) of the Ah receptor.
Activation: cells are exposed to the tested compound alone.
+: weak activity (< 25% of the TCDD maximal response); ++: medium activity (75% > response > 25%); +++: high activity (response > 75 %).
Inhibition: cells are exposed to both the tested compounds and TCDD.
+: antagonistic activity (response < 75% of the TCDD maximal response).
Synergy: response > 100% of the TCDD maximal response.

3. Steroid hormonal pathways (human mammary tumour cells)

Food supplements	*Active ingredients (PC in intestine)*	*ACTIVATION*				*INHIBITION*			
		hAR	*hPR*	*hGR*	*hER*	*hAR*	*hPR*	*hGR*	*hER*
						ppm			
St John's wort	Hypericin (0,5 - 5 ppm)	-	/	-	-	*	/	+ (5,0)	+ (1,3)
	Hyperforin (10 - 100 ppm)	-	/	-	-	*	/	*	+ (0,5)
Ginkgo biloba	Ginkgolide A (0,1 - 7,8 ppm)	-	-	-	-	-	-	-	-
	Ginkgolide B (0,1 - 10,4 ppm)	-	-	-	-	-	-	-	-
	Ginkgolide C (0,4 - 3,1 ppm)	-	-	-	-	-	-	-	-
	Ginkgolide J (0,2 - 2,9 ppm)	-	-	-	-	-	-	-	-
	bilobalide (0,6 - 13,6 ppm)	-	-	-	-	-	-	-	-
	Isorhamnetin (1,3 - 19,2 ppm)	-	-	-	+++ (2,5)	-	+ (2,5)	-	-
	kaempferol (1,3 - 22,4 ppm)	-	-	-	+++ (0,6)	+ (2,5)	+ (2,6)	+ (1,4)	synergy "17b-estradiol-kaempférol"(1,2)
	Quercetin (1,3 - 81,4 ppm)	-	-	-	++ (6,8)	+ (41,3)	+ (6,8)	-	-
	Mix 8 compounds	/	/	/	+++ (6,3)	/	/	/	synergy "17b-estradiol-Mix8"(0,8)
	Mix terpens	/	/	/	-	/	/	/	-
	Mix flavonols	/	/	/	+++ (3,8)	/	/	/	synergy "17b-estradiol-Mix flavonols"(0,5)
	Gingko biloba extract	/	/	/	++ (25,4)	/	/	/	+ (25,4)
Soy isoflavones	Genistein (6 - 92 ppm)	-	-	-	+++ (0,03)	-	+ (5,4)	+ (10,8)	synergy "17b-estradiol-Genistein"(0,03)
	Daidzein (11 - 58 ppm)	-	-	-	+++ (0,1)	synergy "5aDHT-Daidzein"(20,4)	+ (10,2)	+ (20,4)	synergy "17b-estradiol-Daidzein"(0,05)
	Glycitein (10 - 33 ppm)	-	-	-	+++ (2,6)	-	+ (11,4)	+ (19,9)	synergy "17b-estradiol-Glycetein"(1,3)

PC: Plausible concentration; "-" : no effect; " /": not tested ; Number in brackets: minimal concentration showing an activation (corresponding to 15% of the maximal response obtained with the ligand of reference) or an inhibition (corresponding to 75% of the maximal response obtained with the ligand of reference) of the steroid receptor.

* : visual cytotoxicity observed by microscopic examination of the cells.

Activation: cells are exposed to the tested compound alone. +: weak activity (< 25% of the reference ligand maximal response); ++: medium activity (75% > response > 25%); +++: high activity (response > 75 %).

Inhibition: cells are exposed to both the tested compounds and the reference ligand. +: antagonistic activity (< 75% of the reference ligand maximal response).

Synergy: response > 100% of the reference ligand maximal response.

Reference ligands:

hAR: 5alpha-dehydrotestosterone
hPR: Progesterone
hGR: Dexamethasone
hER: 17beta-Estradiol

2.6 Review of the literature about in vitro and in vivo effects of active ingredients

2.6.1 St John's wort

2.6.1.2 Activity on CYP3A4

In vitro

In vitro studies provide variable results. Two studies performed with recombinant microsomes have shown an inhibitory effect on the CYP3A4 activity for hypericin and hyperforin (Obach, 2000). Other studies observe a strong inductive effect of hyperforin on the CYP3A4 protein, for example in human hepatocytes (Komorosky, et al., 2004). Those contradictory results suggest that the hyperforin plays a double role in the CYP3A4 activity. It could inhibit its activity but induce its expression.

In vivo

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; Markowitz, et al., 2003). This inductive effect has been attributed to hyperforin, as No clinically relevant CYP3A induction was observed after St. John's wort with low hyperforin content in healthy volunteers (Mueller et al, 2009).

But taken as monotherapy, St. John's wort has an excellent safety profile (Izzo & Ernst, 2009).

Izzo & Ernst (2009) present an overview of oral drugs that were shown to be influenced by St John's wort intake, mostly by lowering their therapeutic effect:

- on immunosuppressants, such as cyclosporine, used on transplanted patients,
- on cardiovascular drugs, for example warfarin,
- on antiretrovirals drugs (causing treatment failure),
- on anticancer drugs, which sometimes have a narrow therapeutic window,
- on drugs acting on the central nervous system (such as antidepressants, anxiolitics, anti-epileptics and muscle relaxing agents),
- on the gastrointestinal tract and respiratory system,
- on hypoglycaemics, anti-inflammatories, antimicrobials and anti-migraine medicines,
- clinical evidences also suggested that SJW may affect hormonal contraception. Numerous cases of women becoming pregnant while using oral contraceptives and SJW were described (Izzo & Ernst, 2009).

Numerous studies have confirmed the good tolerability of St. John's wort extract and the very low frequency of adverse effects in case of monotherapy. Nevertheless, a change in the metabolism of a drug by the co-administration of another substance like St John's wort food supplements may be clinically relevant. For example, women who take St John's wort concurrently with oral contraceptives should be advised to use other means of birth control or stop using SJW.

2.6.1.3 St John's wort and statins

Statins are the most effective medications for the treatment of primary and secondary hypercholesterolemia. In Belgium, statistics for 2008 show that 8.3% of the Belgian population receives a daily statin treatment, which ranks them as the most prescribed drugs in Belgium (source: INAMI-RIZIV).

Several observations on patients under statin treatment (Gordon et al., 2009), and clinical trials (Sugimoto et al., 2001) show that some statins are significantly less effective when combined with St John's wort food supplements. For example, Sugimoto & al. (2001) observed that St John's wort treatment on healthy volunteers tended to lower plasma simvastatin, but had no influence on pravastatin concentration. Simvastatin is extensively metabolized by CYP3A4 in the intestinal wall and liver, which is induced by St John's wort. In contrast, CYP3A4 has a minor role in the metabolism of pravastatin (Sugimoto, et al., 2001).

Inhibition of the statins efficacy can be critical for health. For example, a 59-year-old man presented to his physician with hyperlipidemia. For his treatment, he received Rosuvastatin 10 mg/day and its total cholesterol blood level decreased to 165 mg/dl. A routine lipid panel was performed 6 months later, and a marked increase of total cholesterol was noted (237 mg/dl). The patient confirmed that he was compliant with Rosuvastatin but had begun taking St. John's wort, 2 capsules per day. He was asked to stop taking the herbal supplement, and a repeat lipid panel 4 months later showed a marked improvement of all parameters (Gordon et al., 2009).

2.6.1.4 St John's wort and cataract

Recent evidence suggests that the St John's wort component hypericin may be associated with cataractogenesis. This association has not been directly evaluated in humans, but a recent epidemiologic study revealed the relationship between self-reported St John's wort use and cataracts utilizing data from a large, population-based sample. The negative effect of hypericin can be explained because of its photosensitivity. It causes the production of oxygen radicals when exposed to visible or ultra-violet light, which bind to the lens crystalline proteins. However animal and human research indicated that photosensitization is not likely to occur at the

recommended dosage levels. Based upon experimental studies (animal and human) it would take approximately 30 to 50 times the recommended daily dose of the standardized extract to produce severe phototoxic effects in humans (Schulz et al. 1998). The results of this study cannot be considered as univoque, but lend support to the hypothesis that St John's wort may indeed increase the risk of cataracts (Booth & McGwin Jr., 2009). It should be evaluated clinically in the future.

2.6.1.5 Health concerns about St John's wort based products: conclusions

St John's wort products have been among the top-selling herbal preparations in developed countries in recent years. The reason for this success is the belief that it is more natural, safer and has fewer or no adverse effects compared to conventional antidepressants (Barnes, 2002). Numerous studies have confirmed the good tolerability of St. John's wort extract and the very low frequency of adverse events in case of monotherapy.

St John's wort is well known for its capacity to interact with drugs. Our *in vitro* results showing that active ingredients of St John Wort are able to modulate the activity of CYP1A1, a xenobiotic metabolizing enzyme, as well as the activity of the Ah receptor, a transcription factor regulating the CYP1A1 gene expression, indicates also possible interactins with xenobiotics. Even if the decreasing effect on the cytochrome CYP1A1 activity by the St John's wort active ingredients in the presence of B[a]P could be considered as positive, this modulation does not take part in the reactions leading to their anti-depressant activity, thus it could induce unknown actions on human health. Moreover, the interactions with the CYP3A4 are dominant and concern an impressing number of drugs used every day by a large section of the population (statins, immunosuppressants, oral contraceptives,…).

For precaution, patients under any drug treatment should not consider SJW as harmless, and thus should inform their physician. Moreover, pharmacists must also give appropriate information. They should be able to recognize the possible interactions and give advice and precautionary measures to the customers. Even when notified, this customized approach is unrealistic for products presented as food supplements for the consumer available in the supermarkets and on the internet. This is an issue, because as we have seen in the first part of the Foodinter project, 48% of the interviewed consumers don't systematically read labels or leaflets ; among these 48%, 17% *never* read labels.

2.6.2 Phytoestrogens

As we have seen previously, soy isoflavones are endocrine disruptors able to play a hormonal role. Their estrogen-like activity is the one responsible for the therapeutic effects when treating menopausal symptoms. The three best characterized phytoestrogens are from soy: daidzein, genistein and glycitein.

Ignored by most consumers, soy is found in more then 60% of processed food. In addition, other phytoestrogens are widely present in the normal human diet, for example in hop, spinach, clover, tea, coffee and rhubarb (AFSSA, 2005). They can also be found in other food supplements than the soy extracts, and have to be added to the phytoestrogens already ingested with normal food, in phytoestrogens risk assessment studies.

The pro and cons of phytoestrogens have been deeply studied.

The attitude of the general public and clinicians toward phytoestrogens (particularly soy phytoestrogens) is generally positive, in opposition to their synthetic counterparts, because of the vegetal origin of the molecules. Moreover, exposure to most synthetic EDs such as pesticides (e.g. DDT and methoxychlor), industrial lubricants (e.g. PCBs) and plasticizers (phtalates and bisphenol A) is frequently associated with alarming statistics regarding declining reproductive health and increasing rates of cancer and obesity (Crain, et al., 2008). Another example of confusion is that in Europe, soy formulations for non breastfeeding infants are available only by prescription and, consequently, the use of soy formulations is customarily lower in these countries. These types of advice are confusing for parents and have done little to quash the idea that soy formulation is a healthful alternative to breastfeeding (Patisaul & Jefferson, 2010). Phytoestrogens remain widely believed to provide an array of beneficial effects, including preventative or therapeutic actions in carcinogenesis, atherosclerosis, menopausal symptoms and osteoporosis.

Studies concerning toxicology of a prolonged oral consumption of phytoestrogens have been conducted mainly in rodents, more rarely on dogs and apes. The results, scattered and contradictory, should thus be carefully considered (AFSSA, 2005).

Nevertheless, it is well established that estrogens promote breast tumorigenesis, and that parameters which increase lifetime estrogen exposure (such as early menarche, short duration breastfeeding, and low parity) are associated with elevated breast cancer risk. Because they bind estrogen receptors with relatively high affinity, some researchers and clinicians are concerned that high phytoestrogen intake may increase the risk of carcinogenesis and put breast cancer survivors at risk for reoccurrence. Others have proposed that the opposite is true, citing traditionally low cancer rates in Asia as evidence (Patisaul & Jefferson, 2010).

The AFSSA concluded in 2005 that studies concerning the cardio-vascular diseases and cancer did not find risks associated to the consumption of isoflavones as a substitute for hormonal treatment. Isoflavones seem associated neither to the benefits (climacteric comfort, osteoporosis) nor to the risks (cardiovascular disease, cancer). Nevertheless, the studies are not numerous, and indicate risks for certain population categories, in particular during pregnancy and early infancy (AFSSA, 2005).

The question of whether or not phytoestrogens are beneficial or harmful to human health remains unsolved. The answer is likely complex and may depend on age, health status, life style and even the presence or absence of specific gut microflora (Patisaul & Jefferson, 2010).

Each phytoestrogen has its own estrogenic potency, compared to the effect of the classical 17β-estradiol. The exact potency profile depends on the *in vitro* material and methods used. The classification is: oestradiol > coumestrol > 8-prenylnaringenin > genistein, and equol > daidzein > glycitein. Data on the oestrogenic potency of mixtures of phytoestrogens are limited. Willard & Frawley (1998) demonstrated that co-treatment with equimolar concentrations of genistein, daidzein, formononetin and equol resulted in an additive oestrogenic response in a reporter gene assay relative to that observed for any individual phytoestrogen. However, Collins *et al* (1997) demonstrated that biochanin A and flavones inhibited the activity of oestradiol in a reporter gene assay, which is predictable for these weak partial agonists (FSA, 2003).

A study performed on rats and measuring different histological parameters leads to the conclusion that most of the effects can be attributed to the estrogenic properties of genistein (McClain et al. 2005). From their data, experts of AFSSA concluded that soy isoflavone intake of up to 1 mg/kg bw/day does not raise safety concerns (AFSSA, march 2005). Genistein has been more thoroughly studied, it is estimated that the level of exposure of genistein for the citizens ranks from 0.016 to 0.833 mg/kg bw/day, depending on their food habits (vegetarians have the highest levels). This is 0,16 % to 83,3% of the safe intake evaluated by the AFSSA. Nevertheless, genistein exposure from isoflavone-containing food supplements may reach 60 mg/day (the claimed benefits may be achieved with daily genistein intakes of 30 - 60 mg for an adult person, corresponding to 1 mg/kg bw/day). Considering a normal diet plus the food supplement intake, the safe daily intake can be widely exceeded. This is clearly a matter of concern.

The Scientific committee of the Belgian Federal Agency for the Safety of the Food Chain (FAFSC) has recommended in 2009 to control the presence of phytoestrogens (more particularly genistein, daidzein and glycitein) in food, with a focus on novel food and food supplements (FASFC, 2009).

Consumers should be aware that soy in food supplements contains endocrine disrupting compounds and make dietary choices accordingly. Consumers must be clearly informed through an adequate labelling. This labelling should mention at least the isoflavone content of the food supplements, the maximal advised intake, and the categories of population that should not consume this product (pregnant women, young children and vegetarians).

Health concerns about Soy isoflavone based products: conclusions

The attitude of the general public and clinicians toward soy phytoestrogens is generally positive in opposition with their synthetic counterparts, because of the vegetal origin of the molecules. A litany of health benefits including a lower risk of osteoporosis, heart disease, breast cancer, and menopausal symptoms, are frequently attributed to phytoestrogens, but many are also considered as endocrine disruptors, indicating that they have the potential to cause adverse effects on health. It is widely documented in the literature, and our observations confirm it: soy isoflavones have an estrogen-like activity and shouldn't be given in high doses to some categories of the population.

As we have seen in this study, the three main soy isoflavones don't induce any stress reaction from bacterial strains. It could be extrapolated to eukaryotic human cells. The decreasing effect of the cytochrome CYP1A1 activity by the three soy isoflavones in the presence of B[a]P could be considered as positive. Nevertheless, this modulation doesn't take part in the reactions leading to their therapeutic activity, thus could induce unknown actions on human health.

The main health concern about the soy isoflavones is their known action as endocrine disruptors. The consumer must be clearly informed through an adequate labelling. This label should mention at least a warning concerning the categories of population that should not consume this kind of product.

2.6.3 *Ginkgo biloba*

Because the *Ginkgo biloba* is a platelet anti-aggregate, hemophiliacs and the people preparing for surgery should avoid taking it. The same advice is to be given for people taking anti-coagulants and anti-inflammatory.

Heath concerns about Ginkgo biloba products: conclusions

With an aging population seeking inexpensive solutions to troubles such as dementia and vasculopathy, *Ginkgo biloba* offers some benefits as a mild vasoactive and neuroprotective phytomedicine. Evidence indicates that it is effective in slowing Alzheimer's disease progression and ameliorating symptoms. The simplicity of the treatment, ease of access and low cost today make *Ginkgo biloba* extract one of the most popular food supplements. However, *Ginkgo biloba* is the second most common herb involved in drug interactions (after St John's wort). The number of reported cases of emerging Ginkgo-drug interactions is already on the rise, and the actual number of cases may in fact be higher due to under-reporting (cited in Abad et al., 2010).

The estrogen-like activity of the flavonoids and the synergy of the mix with a standard activator of estrogen receptors suggest that they may act like isoflavones. Because of their hormonal activity, isoflavones are a matter of concern.

As seen in the previous *in vitro* assays, there is much probable interference between the *Ginkgo biloba* active compounds. It suggests that each food supplement could act differently on the intestinal cells, concerning both cytotoxicity, stress induction, hormonal equilibrium and herbal-drug interaction. The issue is still complicated by the possible presence of environmental contaminants such as heavy metals (founded in two products), and polycyclic aromatic hydrocarbons (excessive levels founded in one product).

2.7 Risk assessment

2.7.1 The sources of risks

According to our observations based on the analyses of 61 products bought in Belgium from supermarkets, specialized shops and internet, and on *in vitro* analysis of active ingredients and mixtures of active ingredients, we have located sources of risks that could have directly and indirectly a negative impact on public health. The sources of risks identified during the Foodinter project can be classified into six major groups: toxicological issues, access to the market, consumer, manufacturing, uncertainties, labeling/advertisement. For each group, the source of risks was either clearly identified, or inspired by the experiences of other countries or from scientific literature. They are summarized here in a Fish Bone diagram (Figure 20).

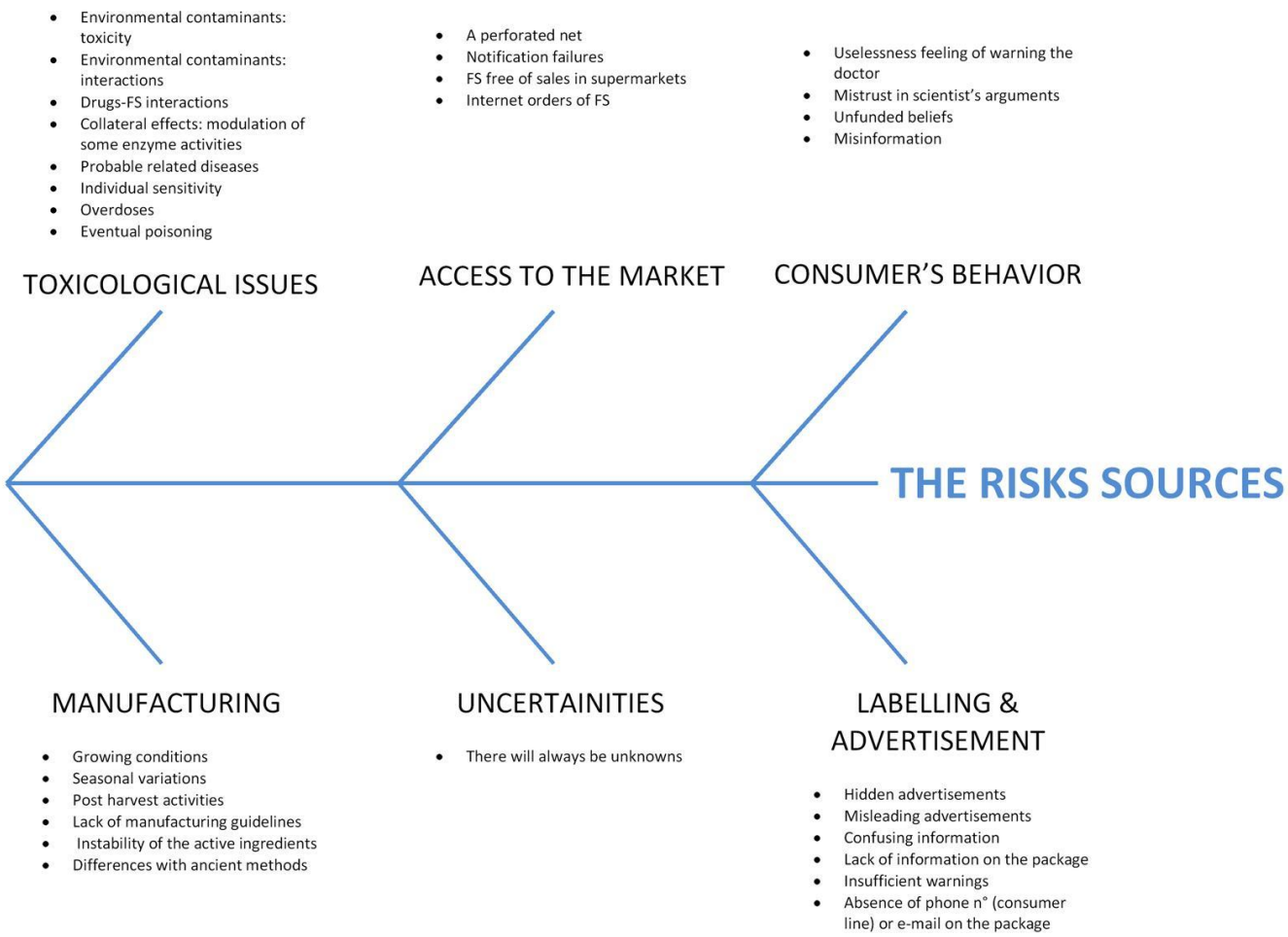


Figure 20: Different sources of risks identified around the plant based Food Supplements consumption.

2.7.2. Toxicological issues

This family of risks refers to the sources of risks that could directly affect human health through its metabolism. The main part of the study concerned the presence of different environmental contaminants, as well as the action of active ingredients and their ability to interact with drugs. The sources can be sorted in three groups. They are:

a. The environmental contaminants

- Some trace elements, like the heavy metals cadmium and lead, were found in some products at concentrations that are too high (health concerns).
- The polycyclic aromatic hydrocarbons (PAHs) were found in some products at high concentrations. S^{t}-John's wort and *Ginkgo biloba* extracts presented the most frequent contaminations and the highest average values for PAHs concentrations.

- Environmental contaminants such as mycotoxins, heavy metals, antifungals, PAHs, etc. :
    - may modulate the activity of the CYPs (proteins implicated in the metabolism of various drugs),
    - may modify the activity and the expression of efflux pumps, like the P-glycoprotein (Pgp). Intestinal Pgp is a versatile multi-substrate pump, which can expel compounds out of the cells in order to bring them back to the luminal side of the intestinal barrier and maintain intracellular concentrations at low levels. The role of Pgp in drug interactions and bioavailability has been highlighted.

b. <u>The toxicity of the active ingredients</u>

- Possible related diseases are suspected to be provoked, like cataracts with S^t-John's wort consumption,
- Over dosage: some products are more concentrated in active ingredients than the legal limits (found in some S^t-John's wort products),
- Poisoning: deaths through poisoning following consumption of such products is unprobable as consumption of food supplements is most often limited in amounts,
- Each person has its own sensitivity to the ingredients ingested. The reasons are to be found in the genetic profile -this might explain the different responses to soy isoflavones between Caucasian and Asian people- and/or in the gut microflora -each individual's gut microbiota affects the bioavailability and response to orally administered drugs.

c. <u>The interactions due to the active ingredients</u>

- Interactions between drugs and some food supplements based on plants are well known by the scientific community. In our study, we have seen that some active ingredients modulate the activity of cytochromes, that are proteins that metabolize drugs,
- There are possibilities of collateral effects of some FS and their active ingredients, for example through the modulation of the cytochromes CYP1A1 activity, the interference with the dioxin pathway, and the endocrine disruptor effects.

2.7.3. Manufacturing

Some results of our study suggest that the manufacturing could influence the quality of the products, in terms of environmental contaminants (such as PAHs or heavy metals) or active ingredients concentration :

- Growing conditions, often widely different from the traditional conditions, can be at the origin of high variation in the chemical content,
- Seasonal variations can also explain differences in the quality and concentrations of the active ingredients,
- Misidentification of plants harvested from the wild is also a continuing problem,
- The concentration of active ingredients depends on the organs of the plant, it is important to provide the exact plant part of the extract,
- Post-harvest activities, like transport and storage of the raw material, can be at the origin of contamination (for example with lead and PAHs), and development of mycotoxins,
- The manufacturing methods are sometimes different from the ones that were used traditionally. The concentration of non-desirable substances can thus increase. This has been observed for maca-based products, for which the traditional preparation would decrease the presence of some dangerous compounds,

In conclusion, it is important to make autorities aware that controls must be ensured at batch level and to check if the manufacturing conditions are taken into consideration in the HACCP and autocontrol procedures of the producer.

2.7.4. Access to the market

- Notification is required for any food supplement that is meant to reach the Belgian shops (pharmacies, supermarkets, specialized shops…). Nevertheless, out of 25 products bought in the above mentioned places, 11 were not authorized to be on the Belgian market at that moment. This means that some products pass the net and reach the market's stands. Controls were performed in 2007 by the FASFC on more than 5000 products; they had similar results,
- There are sometimes notification failures; in our study two products showed deficiencies in labelling. It also happens that new (and illegal) health claims are written on the packages, which were not announced in the administrative file. The problem was worrying enough for the FASFC to perform specific actions on food supplements,

- The quantity of internet orders of food supplements is still not known, but it is obvious that it will grow during the following years. This market is totally out of control: only 4 of the products bought on the internet were notified (out of 36),
- Products are free for sale in the supermarket, which is obviously criticized by the pharmacists who consider important to provide advice to the consumers. It can also be argued that, in the frame of this study, supermarkets have more frequently unauthorized products in their stands.

2.7.5. Consumer's behavior

The consumer's behavior remains problematic in some aspects.

- They feel unnecessary to warn the physician about their consumption, probably because it is free for sale in supermarkets, thus considered as a simple food,
- Consumers mistrust the scientist's arguments, sometimes because they consider that scientists undergo lobbying,
- Consumers share some unfunded beliefs, considering for example that the food supplements based on plants are de facto natural and safe. 18 to 45% consider the food supplements as perfectly safe.
- Misinformation reigns in the mass-media society. The lack of criticism increases because of a "crowd effect".

2.7.6. Labeling/advertisement

- Hidden advertisements, like "publi-reportages", statements, or even journal articles, are spread in newspapers and magazines.
- Misleading advertisements tend to overstate the effects of the product, for example with comforting pictures. It creates confusion in the consumer's mind.
- Clear health claims appear on advertisements. *"In December 2006 EU decision makers adopted a Regulation on the use of nutrition and health claims for foods which lays down harmonised EU-wide rules for the use of health or nutritional claims on foodstuffs based on nutrient profiles. …. One of the key objectives of this Regulation is to ensure that any claim made on a food label in the EU is clear and substantiated by scientific evidence » (statement on Nutrition and Health Claims from the EFSA website) ,* Of course we are still in a transition period during which the food supplement sector have to adapt their communication to the consumers There is also often insufficient information on the labels (like a phone number for consumer's),
- Warnings are sometimes insufficient on the labels.

2.7.7. Uncertainties

There will always be unknowns; it is clearly unfeasible to gain detailed knowledge about all the effects in a short time-span. However, the lack of a clear path forward cannot be an excuse for standing still. Each piece of scientific data is a part of a complex puzzle.

Because each food supplement is a complex mixture of active ingredients, plant constituents and metabolites, it is obvious that they are completely different from the classical drugs sold in pharmacies. For a better understanding and control of their potential health impacts, it is pertinent to study the food supplements "*from pure to sold*": first each potential active ingredient in a pure standard form with realistic intestinal concentrations, then mixtures of those active ingredients, then standard extracts (if available), and finally crude food supplements as they are sold. Since this will be in practice impossible to perform for each FS to be put on the market, there will always be uncertainties about potential effects related to the complex composition of the mixtures.

3 POLICY SUPPORT

3.1 Recommendations based on the scientific observations

3.1.1 Major health concerns

The weight of each risk source depends on the probability for the occurrence of harmful effects on human health and the severity of that harm. Because the health issues are widely depending on the consumption behaviours, the assessment cannot consider only the scientific observations but also sociological information and labelling information.

For public health, there are three major risks linked to the intake of plant based food supplements: the interactions with numerous drugs, which may modulate their efficiency, environmental contaminants such as heavy metals and polycyclic aromatic hydrocarbons, and finally biological effects of some active ingredients, acting as endocrine disruptors for example. There are several ways to decrease the negative impact of plant based food supplements.

3.1.1.1 Interactions between food supplements and drugs

To decrease the health concerns around the drugs-food supplements interactions, it is imperative to encourage all the health professionals (doctors, pharmacists) to alert their patients when prescribing drugs. Even in each variety of plant, depending on the manufacturing, there might be unexpected interactions. The precautionary principle should be encouraged.

In parallel, everything must be done to increase the patient's awareness to inform their physician of their eventual consumption of food supplements. People should be aware that those products are not harmless and that natural products are not in any event safe. A recent survey performed by the Eurobarometer concerning the consumer's perceptions of food-related risks mentioned that the Belgians express a high level of confidence to their physician/doctor and health professionals (93%), significantly higher than any other information source (TNS Opinion & Social, 2010).

Finally, new scientific studies should be encouraged in order to understand more precisely which are the modes of action of the food supplements and their active ingredients.

These two issues (communication and scientific knowledge) have been addressed in this FOODINTER project, as illustrated in Figure 21.

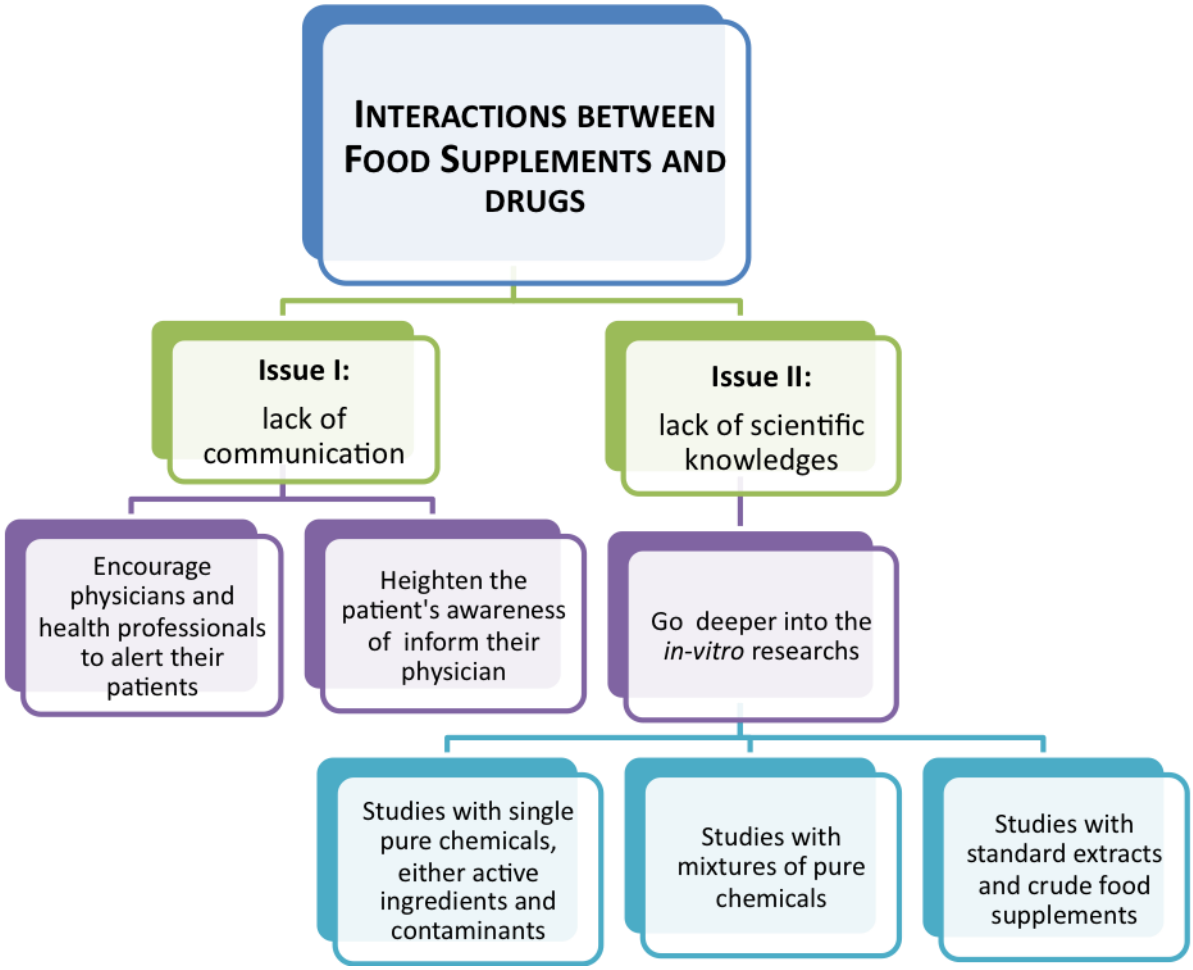


Figure 21 : Recommendations regarding interactions between FS and drugs.

3.1.1.2 Environmental contaminants and toxicity

The struggle against contaminations should be already performed at the beginning of the production. Clear guidelines should be proposed to the producers in order to avoid contaminations. They should cover all the steps: from growing conditions to transport and conservation, following the HACCP (Hazard Analysis Critical Control Point) approach (which is mandatory according the European Regulation (CE) 852/2004) in order to detect and manage the critical points. For lead and PAHs, Codex alimentarius international codes of practice for the prevention and reduction of these contaminants should be followed (http://www.codexalimentarius.net/web/standard_list.do?lang=en).

Environmental contaminants 'heavy metals' and 'polycyclic aromatic compounds' contents should be systematically verified prior to notification.

Randomized controls have already proven their efficiency in terms of wild presence of not notified products on the market, of non-compliant labels, and of

some contaminations. During 2009 the FASFC performed a new control campaign similar to the one in 2007. Effective improvements were observed, thus the controls are efficient. Those controls should be developed in order to follow the exponential growth of this market.

In parallel, targeted controls should be organized according to the specificity of the food supplement.

- If the manufacturing requires specific drying steps, the focus should be on PAHs and lead content.
- If the storage conditions are suspicious, a focus should be on mycotoxins content.
- If the supplement is based on animal fat (e.g. omega 3 in fish oil), the focus should be on dioxin and PCB content.
- If the geographical origin of the plant is suspicious, a complete battery of test should be envisaged.
- According to the claimed health benefit, some specific tests should be envisaged in order to find eventual illegal drugs.

The struggle against internet sales of uncontrolled products will probably be the next challenge for the public authorities. People should be informed that internet is not a safe source of food supplements.

Several exotic plants, like the Maca root (*Lepidium meyenii)*, generate debates around their efficiency and toxicity. The mode of action of Maca roots is unknown. Regarding the lack of knowledge on Maca root and its active ingredients, it could be envisaged to exclude this product from the Belgian market. The situation is unclear: Maca has substantial medicinal use in traditional herbal medicine by indigenous cultures, with a specific protocol to prepare it. The presence of alkaloids measured in this food supplement can be explained by the industrialization of the preparation of the products. Alkaloids are an indicator of a wrong process. Because they are known to be harmful, no residue of alkaloids is permitted in the food supplement. On the other side, those alkaloids are markers of authenticity, thus permit to diagnose false Maca extracts.

Scientific *in vitro* tests suggest that Maca might lead to health concerns. The EFSA mentioned the maca in the list of "plants reported to contain toxic, additive, psychotropic, or other substances of concern". The EFSA clarifies that this list does not imply that those plants are not safe for use in food supplement plants, but should be subjected to a more extensive safety assessment. A better understanding and characterization should be awaited before letting those products reach the Belgian market.

As advised previously by a European scientific committee (EFSA, 2004), as purity specifications for all botanicals and botanical preparations are very difficult to define, the development of *ad hoc* manufacturing guidelines could be considered in

order to improve their characterization and safety. The EFSA published recently a guidance document for that purpose (EFSA, 2009).

Figure 22 summarizes the issues linked to the question of contaminants in food supplements.

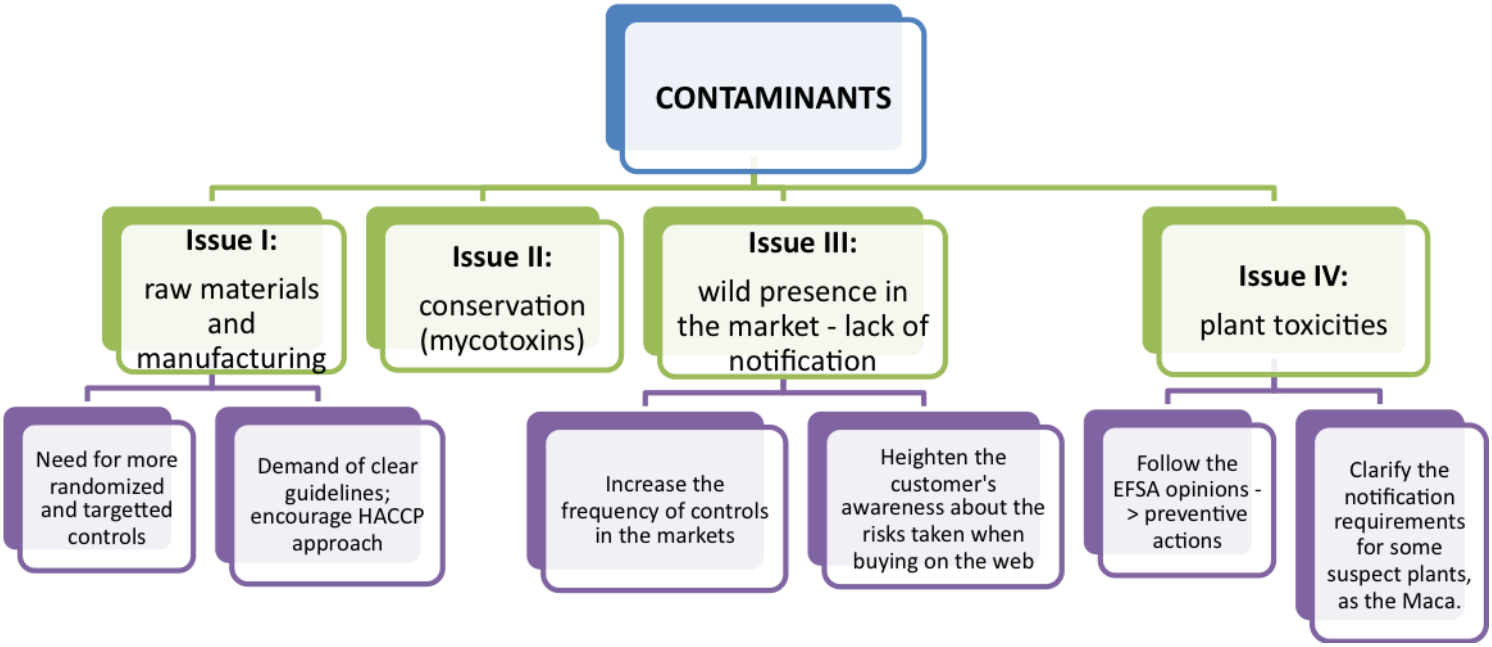


Figure 22 : Recommendations regarding the issues linked to the presence of contaminants in food supplements.

3.1.1.2 Biological activities

During this study, we have confirmed that some active ingredients may act as endocrine disruptors (in Soy isoflavones and *Ginkgo biloba*). More precisely, they have an estrogen-like activity and are thus called phyto-estrogens. This property is the reason for the claimed therapeutic effects of Soy isoflavones: reduction of menopausal symptoms. Nevertheless, phytoestrogens are known to be harmful for some categories of population that should be clearly informed (pregnant women, young children and vegetarians). Considering a normal diet plus the food supplement intake, the safe daily intake can be widely exceeded.

Other biological effects are to be mentioned, such as possible side effects, overdoses, and individual susceptibilities. For all those problems, a good information campaign to the consumers should be planned in order to make people aware of the fact that food supplements are not harmless.

Figure 23 summarizes the issues linked to the question of biological activities of food supplement active ingredients.

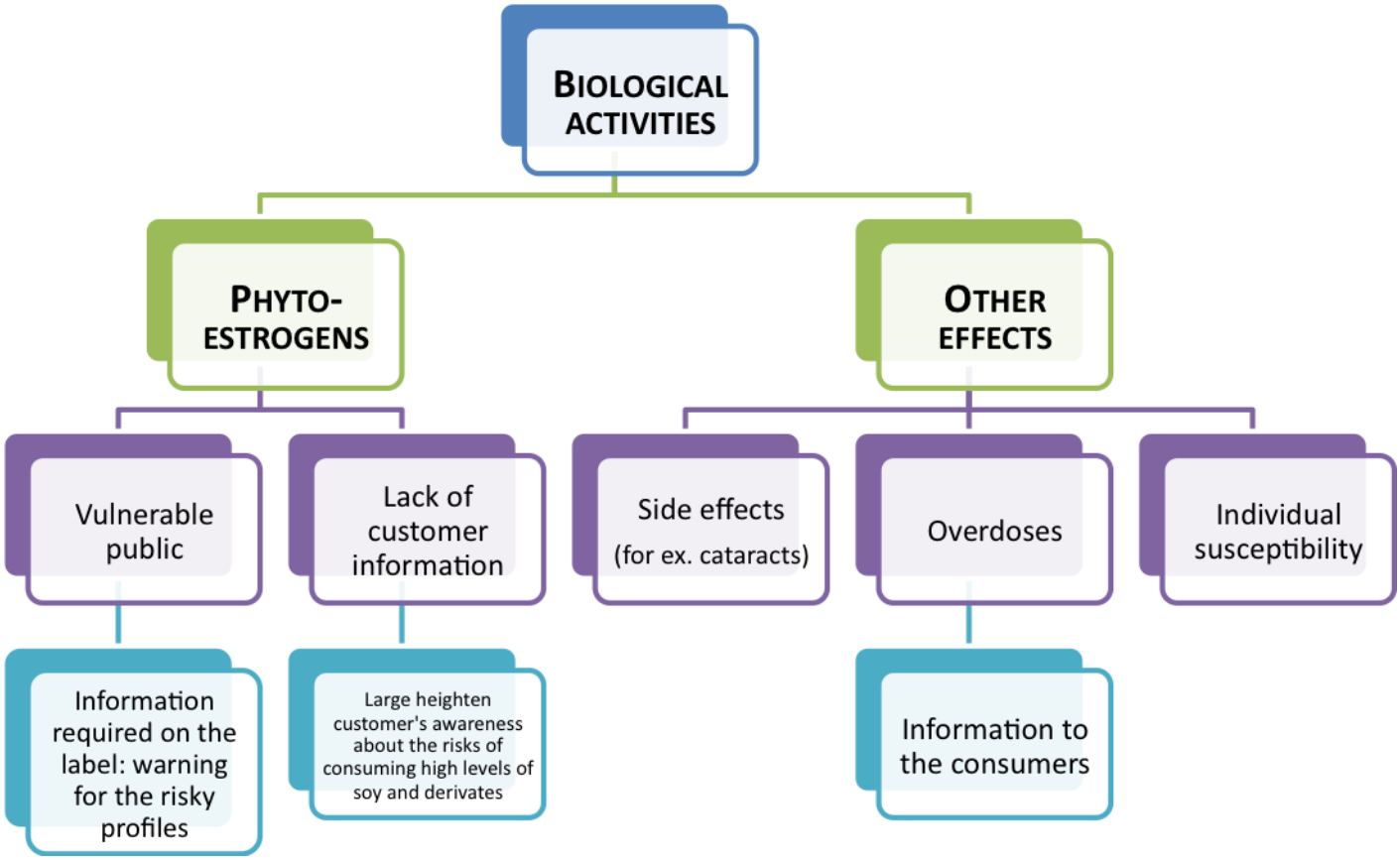


Figure 23 : Recommendations regarding the issues linked to the potential biological activities and/or toxicity of food supplements ingredients.

3.1.2 The "Tradition-based claims": a pervert argument ?

When defending their products, it is frequently argued by the industrials that the traditional knowledge about the use and efficacy of botanical preparations should not be denied. Arguments cover the fact that knowledge and experience has been accumulated by mankind "over thousands of years" by carefully observing the properties, by optimizing their use, before the age of clinical trials and sophisticated methods.

This opinion can not be shared in face of the observations of the Foodinter project:

- Even if used for millenniums, the fact that direct side effects haven't been observed does not guarantee that there are no recorded health issues, which could not be directly related to the plant consumption. For example, as mentioned in this report, St-John's wort supplements are seriously suspected to induce cataracts. Many side effects of plant food supplements are probably still to be discovered.
- The traditional manufacturing process is far from the mass production that we currently meet. As cited by the EFSA in 2009, botanicals and botanical

preparations might become hazardous as a result of deviations in the production process (e.g. misclassification, switching of species).

- In opposition with the industrial process, the traditional manufacturing might also eliminate toxic compounds (the Maca root for example), and on the other side, the industrial manufacturing can induce their appearance. As the EFSA, we conclude that the safety of botanicals and botanical preparations should be ensured by following a Hazard Analysis and Critical Control Point (HACCP) approach. It must be applied with the necessary flexibility and adapted to each botanical preparation on a case-by-case basis.
- Plant based food supplements are taken in concentrated doses (in opposition to traditional infusions (tisanes)), for example. The intake of active ingredients might be significantly higher that the estimated historical intake level. The impact of those high intake levels cannot be assessed only on the basis of the "historical experience".

3.2 Reflexions and recommendations for risk communication based on the sociological tasks

3.2.1 Differentiate communication strategies according to the heterogeneity in consumer profiles and FS consumption patterns

When presenting the results of sociological tasks, we underlined the diversity one is to face when analyzing FS consumption and FS consumers. If one wants to diminish the risks associated with FS consumption, then it's thought that consumers have to be helped to put themselves or their consumption in perspective, and to wonder whether FS are a solution to the "problems" they face. In order to do that, we have to admit that it requires to touch consumers at the centre of their practices and framings, at the centre of what they consider a problem, a risk or a lack in their lifestyle, that justifies their consumption. Therefore, the rhetoric at work behind the assessment by consumers themselves that they "have a problem" (or could avoid some) should also be addressed ; not the product itself, but the discourses and societal evolutions that give them a grip on consumers : "I could be stronger" ; "I could be less stressed" ; "I could reinforce my immune system" ; and so on.

We underlined that it would be very difficult to address FS consumers in their globalism (moreover regarding the often very specific and contextual nature of risks

related to FS and FF), and that a multifaceted, tailored risk communication and risk management strategy would be a more suitable answer. This would be much more talkative to consumers, and would suit their framings of these diverse "patterns" or "models" we identified in part 2.1.3. of this report, by reinserting in its "context" (or with shared references) the message that is to be heard (and analyzed) by consumers. This message should take into account the fact that there are indeed a lot of objectives that consumers pursue through FS consumption, each having to be addressed in its specificity.

For instance, consumers using FS on the "preventive" mode (that is more rooted in familial references and "traditional" categories) seem "educated" to reason in terms of purpose, and are not familiar at all with categories of legal status or of active substances ; this would expose them to too much complexity and blur their knowledge-building process. We could give another example of a sportsman, that consumes FS in order to boost some of his performances, and that has undertaken a lot of research to manage to understand the working principles of products, their composition, their long term effect, etc., for whom a more "expert" communication could be suited, or a communication that would be relayed by its sport trainer (or sport center), or sport-FS retailer.

This also raises the need for the communication strategy to be tailored to the various networks of advice that appeared from the focus groups and surveys (corresponding to the "profiles" discussed in the results) : these can be informal channels (friends, family, web sites, sport trainers, advertising and articles in reviews, …), as well as formal channels (practitioners, pharmacists, "alternative therapists" (whose status should be clarified), …). No hierarchy or dismiss should be done between them, as consumers don't do hierarchy between those channels neither, except on the basis of trust. This is why they can give more weigh to trusted relatives, or their self-judgment and feelings, than to the advice of practitioners, even if they don't have the competence.

3.2.2. Internet-based risk communication, risk deliberation, and risk governance platform

It was discussed with consumers about the idea of a web site that could give them "good information" on FS products and associated risks. We imagined a multi-level communication tool or "multi-purpose platform", with different parts accessible according to consumers' or other actors' demands or needs. Some parts or features could also be restricted to certain specialists or health professionals, as we will discuss further.

The first level of information could be a traditional form of risk communication, where consumers could find good practice rules (tips and recommendations, like

"don't buy this brand/product", "don't buy on this website", "don't use this FS wit this food", ...), or general assessed information classified by products, and designed to suit their framings or "profiles". We can also imagine presenting precise examples of the risks that are warned against, which are generally very striking to consumers.

The need for "good information" was strongly underlined by consumers, who get often drown into the ocean of (mainly unverified) information they can find. "Good" information was defined as clear, independent/impartial/unbiased, complete or making its limits explicit. This leads to the importance of the second level of information that should be developed on the web site : more than only tips or advice, we think that this tool could be of major interest in order to allow consumers' empowerment, that is giving them the keys : (1) to distinguish between product categories in the mess of "alternative health products" ; (2) to identify and understand the risks associated with FS and FF (and extensively with other assimilated products), as well as the way they are managed, and the place or roles consumers could have in face of these risks. Accordingly, consumers should be given neutral, realist information and helped to make their own opinion and judge if they should adapt their consumption practices. If one wants consumers to take their responsibility in face of risks associated with a consumption they chose, then they should be given the means, knowledge and critical distance to take this responsibility !

A third dimension of this web site should allow discussion with consumers. We already mentioned that it's important to consider the variation in consumers' profiles and patterns of consumption (so not targeting only one, "mean" target, but different ones ; also, communication should be done on various level of complexity, to adapt to the various expectancies and capacities of understanding of consumers), what is a first step towards a two-way communication process. But the idea of implementing a discussion platform (an Internet "forum") would go beyond this and be a much more radical step towards real dialogic governance, allowing consumers to become actors in different ways and not only receptors or targets whose behaviour is to be changed. Moreover, this would be an initiative that would respond to consumers' expectancies, as existing forums are very popular (but on which the quality and independence of information is a huge problem.

This forum could be subdivided in different parts :

– one where consumers could discuss with other consumers or consumers associations (like it happens on the majority of Internet forums, like for instance "Doctissimo") ; but the forum we imagine should be different that the one we can find presently, as we plead for its explicit moderation by scientists, therapists and risk or products experts. This would be great importance, as aiming at preventing from giving unproven or partial advice. However, the idea would be that experts shouldn't need to answer in person to each question, as other members of the forum (consumers) could give suited answers too ;

these would only have to be monitored and verified or balanced. These discussions, like what happens on forums, could become references for other consumers having similar questionings

- one where consumers could ask questions directly to specialists (from the discipline they look for : formal medical disciplines, "alternative medicines", risk experts, regulation or management experts, …)
- one where consumers could formulate remarks or recommendations on products and risks management, as well as on risk communication (what they don't understand, what they don't agree with, …) ; this could be very helpful for the designers of communication or management strategies, assuming that every remark couldn't be taken into account but that the designers (experts) should be open to the idea that "simple" citizen directly voice out concerns to them...and of course have the will to listen and give answers to these concerns.

A fourth part of the website would detail and explain the basis of the risk management system, in a way that makes explicit the presuppositions and limits of this system (namely uncertainty (i.e. long term effects of products), complexity (due to huge number of products, high pace of evolution, and large scope of risks (depending on potentially unlimited factors and interactions). This would allow consumers to increase their knowledge on the reality of risk management, and put back the "myths" or ideals behind risk management in perspective. To sum, this would increase the reflexivity of consumers and allow them to better understand what are the real challenges in risk management and which behavior they have to adopt in face of them.

A fifth part could allow discussion and sharing of knowledge on risks, between scientists/experts and between health professionals on (on the mode of scientific reviews, but also on a "simplified" mode, giving summaries or analyzing controversial issues). This would be a platform for the state of knowledge on risks associated with FS consumption substances, environmental contaminants, interactions, …). We can also imagine a kind of "forum" dedicated to risk experts, scientists and health professionals, where they could ask questions or share ideas and knowledge on FS.

In addition, it could give precious information to those experts and health professionals on consumers' practices or opinions, perceptions of risks, reactions to or incomprehension of risk communication

At least (but not at last, as this tool should be open and in constant evolution), a sixth part would be dedicated to social sciences research on risk communication and risk management. Indeed, the totality of information published by the members of the forums (consumers or experts) would be precious material to analyze, in order

to help increase the reflexivity of the whole risk governance system, that has to be adaptive !

Briefly, we then identified some limits of this Internet tool :

- every consumer may not have Internet, or may not know about the website ; accordingly, other media should be used, such as folders to let in "hot spots" (drugstores, at therapists', in sport centers, …), publication of articles in popular reviews, of advertisements, TV spots (on the mode of documentaries rather than only (too) short spots, even if both would have advantages and disadvantages), …

Accordingly, the second level of information we identified should also aim the media and resources (reviews, web sites, advertisements, ...) that presently touches consumers, that they can be using to guide their FS consumption

- this website would have to become popular, which would take time and implication from public actors as well as from the experts that should participate in the web site management ; it won't become a widely shared reference in one year
- if it becomes popular (but the means should be allowed so that this happens to be), however, it will certainly become subject to attacks from interest groups and lobbies, for whom reflexivity, critic and consumer empowerment is seldom encouraged

3.2.3. Recommendations concerning health professionals (and more generally the healthcare system)

Consumers pointed out the lack of knowledge from general practitioners on nutrition and "alternative health products" or "alternative therapies", as well as interactions between all those compounds.

This lack of knowledge from practitioners could also be a lack of will or interest in alternative therapies, or even a strategy of defence of professional interests, as these alternatives to conventional medicine are often dismissed by doctors.

But this could also be a lack of time or capacities, as we can't expect from practitioners to know everything on every products or interactions. Moreover, the conventional form of medical consultation, driving the approach of practitioners, is rooted in a "problem-solution" approach, often simplified or routinized, to which should correspond a specific drug. When patients would want to analyze complex interactions, then practitioners show generally a lack of will or knowledge to inquire complex interactions, and send them back to specialists'. This sends to another problem, which is that consumers can't always afford these specialists, and will assuredly often prefer cheap self-research and auto-medication in this case.

This underlines the need to improve the basic training and formation of practitioners (but also of herbalists or other therapists or advisers) on nutritional aspects, on "alternative health products" and on interaction risks. All these have become a widespread reality they can't ignore nor dismiss anymore.

They have to be pushed to study complexity in its depth (and ask a large amount of questions, on food, FS consumption, habits, …), to take time to formulate precise, tailored advice, without necessarily being sent to specialists (or else consultation of specialists should be partly refunded by the healthcare system).

3.2.4. Increase the quantity and quality of the information displayed by producers

This call for more and better information aimed at better labels (more information on contents, concentrations, origin of compounds, …) and obligation of notices of use (detailing interaction risks, but also the testings having been conducted and their limits), certification schemes passed (and what they assess), …

For certification, a clear sign should be displayed on the label so that the product could be quickly identified by consumers when buying the product somewhere else than in drugstores. We could also imagine a training to be followed and passed by producers on risks associated with FS ; like for certification, a picture could easily assess that these formations have been passed.

NAREDI, the association of food supplements producers, as a stakeholder in the National Food and Health Plan (http://www.health.belgium.be/eportal/Myhealth/Healthylife/Food/FoodandHealthPlan/index.htm?fodnlang=fr) recommended already in 2008 the concerned Working Group headed by Professor Jean Nève to create a brochure explaining Food Supplements to the Belgian population. This initiative should be encouraged by the authorities and finalized as soon as possible.

3.2.5.Improve the clarity and efficiency of food supplements (risk) management and procedures, defined through European and Belgian regulations and administration

As risk management procedures were not well understood by consumers, they formulated a call for clarification of these :

– What does "notification" assess ? (Does it include controls of compounds ? Additional testings ?) ; Why not making systematic testings of every products,

if we want this procedure to be really safe and not only a *"procedure for the pleasure of procedure"* ?

- What cover the various categories of products created by law correspond ("MP", NP", "NNP", "herbal products/plants", "other products", …) ? What do they mean, what are the differences between them, and to what does it correspond in the whole health products range ? Is it not a way to make different constraints on producers or networks of retailing (especially the concern of herbal products, which are about to be banished from herbalists') ?
- Is the control efficient and trustful, as only very few people work on it at the federal agency and are supposed to control every product on the market ?

Moreover than being criticized as unclear and insufficient risk management, it was criticized by consumers that some non-notified products could be easily be found on the Belgian market, raising the important question of the efficacy of the procedures, and the consequent (lack of) trust consumers have in them.

But more than solely "adding more control" – a model whose limits are obvious in our era of complexity and uncertainty, combined with high expectancies from the public –, shouldn't we also find new paradigms and procedures to both define and manage risks and the (still) unknown ? Those procedures and their design should be more opened up to discussion and co-elaboration with the public and professionals, as well as be more adaptive and reflexive...what is especially challenging in a context of high industrial or professional lobbying.

Nevertheless, it must be recognized that Belgium has a leading role in the European Union in the field of food supplements. The FASFC organized during the Belgian presidency a symposium on our Belgian self-checking systems. Among these systems, the guide G-011 for the FS sector is an officially approved self-checking guide as reference tool for the manufacture of food supplements in Belgium.

4 DISSEMINATION AND VALORISATION

A workshop has been organized on 23 March 2011 afternoon, in Tervuren, open to the scientific community and the representatives of Belgian authorities, as well as to representative of food supplements producers (see program in annex 4).

5 PUBLICATIONS

Danyi, S., Brose, F., Brasseur, C., Schneider, Y.J., Larondelle, Y., Pussemier, L., Robbens J., De Saeger S., Maghuin-Rogister G., Scippo M.L. (2009). Analysis of EU priority polyciclic aromatic hydrocarbons in food supplements using high performance liquid chromatography coupled to an ultraviolet, diode array or fluorescent detector. Analytica Chimica Acta 633 , pp. 293-299.

Diana Di Mavungu J., Monbaliu S., Scippo M.L., Maghuin-Rogister G., Schneider Y.J., Larondelle Y., Callebaut A., Robbens J., Van Peteghem C. and De Saeger S. (2009) LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment. 26:885-895.

Njumbe Ediage E., Diana Di Mavungu J., Scippo M.L., Schneider Y.J., Larondelle Y., Callebaut A., Robbens J., Van Peteghem C., De Saeger S.. Screening, Identification and Quantification of glucosinolates in black radish (*Raphanus sativus L. niger)* based dietary supplements using liquid chromatography coupled with a photodiode array and liquid chromatography – mass spectrometry. Journal of Chromatography A, 1218 (2011) 4395-4405.

Ribonnet L., Callebaut A., Nobels I., Scippo M.-L., Schneider Y.-J., De Saeger S., Pussemier L. and Larondelle Y. (2011). Modulation of CYP1A1 activity at the intestinal level by a *Ginkgo biloba* extract in the human intestinal Caco-2 cells. Toxicology Letters 202: 193-202

6 ACKNOWLEDGMENTS

The promoters of the FOODINTER project would like to acknowledge the Belgian Science Policy (Belspo), for funding the project, the members of the follow up committee for their advices, as well as Christine Mathieu (from Belpso).

7 BIBLIOGRAPHY

Abad, M., Bedoya, L., & Bermejo, P. (2010). An update on drug interaction whith the herbal medicine *Ginkgo biloba*. Current drug metabolism , 171-181.

Abelson J. & al., (2003), "Deliberations about deliberative methods: issues in the design and evaluation of public participation processes", in Social Science & Medicine, Vol.57, ed. Pergamon, pp. 239–251.

AFSSA (2004). Saisine n° 2004-SA-0155 . AVIS de l'Agence française de sécurité sanitaire des aliments relatif à l'évaluation du risque pour la santé du consommateur de l'emploi de la poudre de racine de maca, commercialisée telle quelle ou entrant dans la composition de compléments alimentaires

AFSSA (2005). Sécurité et bénéfices des phyto-estrogènes apportés par l'alimentation - Recommandations.

Albertine, S., Oetterer, M., & Prado Filho, L. (1997). Source of contamination. Boletim da SBCTA 31 , 137-147.

Baars, A., & Theelen, R. (2001). "Re-evaluation of human-toxicological maximum permissible risk levels. On line at:

http://www.rivm.nl/bibliotheek/rapporten/711701025.pdf. RIVM report 711701025

Bailey, D. and Spence, J.D. (1991). Interaction of citrus juices with felodipine and nifedipine. Lancet 337(8736) : 268-269.

Barnes, J. (2002, June 29). Depression. The Pharmaceutical Journal , pp. 908-910.

Beck U., (1986), *La société du risque. Sur la voie d'une autre modernité*, Paris, Éditions Aubier, 2001 ; édition originale : *Risikogesellschaft*, Francfort, Suhrkamp Verlag, 1986.

Beck U., (1991), *La politique dans la société du risque. Essais et analyses*, Suhrkamp Verlag, Francfort-sur-le-Main.

Beck G, Cropp C., (2008), "Infrastructures of Risk: An ANT Approach towards Controversies of Risks", ISA Forum of Sociology, Barcelona, September 5-8, 2008, Thematic Group on Sociology of Risk and Unceartainty TG04, 13p.

Beck, G., & Kropp, C. (2010). Is science based consumer advice prepared to deal with uncertainities in second modernity? The role of scientific experts in risk communication in the case of food suplements. Science, Technology & Innovation Studies (forthcoming), 24 p.

Bender D.E. and Ewbank D., (1994), "The focus group as a tool for health research: issues in design and analysis", in Health Transition Review, Vol 4 n°1, pp. 63-79.

BfR (2008). What information do consumers need about food supplements? Available online : http://www.bfr.bund.de/cd/25170. Consulted on 15 January 2011.

Booth, J., & McGwin Jr., G. (2009). The association between Self-Reported Cataracts and St John's wort. Current Eye Research , 863-866.

Breithaupt H., Gannon F. and Hadley C., (2004), "Degrees of risk", in European Molecular Biology Association reports, Vol. 5 (Special Issue), preface, pp. 1-3.

Brera, C., De Santis, B., Debegnach, F., & Miraglia, M. (2008). Mycotoxins. in B. Barcelo, Food contaminants and residue analysis (pp. 363-427). Elsevier.

Brown, S. (2009). The New Deficit Model. Nature nanotechnology, 4 , 609-611.

Cahagnier, B. (1998). Moisissures des aliments peu hydratés. Paris: Ed. Lavoisier.

Calabrese E. J., (2004), "Hormesis: a revolution in toxicology, risk assessment and medicine. Re-framing the dose–response relationship, in European Molecular Biology Association reports, Vol. 5 (Special Issue), pp. 37-40.

Callon, Lascoumes P. and Barthe Y., (2001), "Acting in an uncertain world : an essay on technical democracy, éd. du Seuil, 298p.

Chavez, M. L., Jordan, M. A., & Chavez, P. I. (2005). Evidence-based drug-herbal interactions. Life Sciences , 2146-2157.

Choi, E., & Kim, G.-H. (2008). Daidzein causes cell cycle arrest at the G1 and G2/M phases in human breast cancer MCF-7 and MDA-MB-453 cells. Phytomedicine 15 , 683–690.

CIAA, 2010, "CIAA welcomes the EFSA Workshop on Health Claims", press release, 6th December 2010, Brussels, 1p.

Ciska, E., Martyniak-Przybyszewska, B., & Kozlowska, H. (2000). Content of glucosinolates in Cruciferous vegetables Grown at the same site for two years under different climatic conditions. J. Agric. Food Chem , 48, 2862-2867.

Clemens, S. (2006). Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie , 88, 1707-1719.

Colalto, C. (2010). Herbal interactions on absorption of drugs: Mechanisms of action and clinical risk assessment. Pharmacological research , doi:10.1016/j.phrs.2010.04.001.

Collins B.M., McLachlan J.A., Arnold F.A. (1997) The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. Steroids, 62, 365-372.

Conseil Supérieur de la Santé (2009). Recommandations nutritionnelles pour la Belgique (révision 2009). Avis n° 8309. Available online http://www.health.belgium.be/eportal/Aboutus/relatedinstitutions/SuperiorHealthCouncil/index.htm.

Conseil Supérieur de la Santé, (2009), "Avis du Conseil Supérieur de la Santé n°8592 : Révision des recommandations concernant un apport complémentaire en lycopène et en lutéine dans les compléments alimentaires", 14p.

Costa, C., Catania, S., De Pasquale, R., Stancanelli, R., Scribano, G., & Melchini, A. (2010). Exposure of human skin to benzo[a]pyrene: role of CYP1A1 and aryl hydrocarbon receptor in oxidative stress generation. Toxicology 271(3) , pp. 83-86.

Crain, D., Janssen, S., Edwards, T., Heindel, J., Ho, S., Hunt, P., Iguchi T., Juul A., McLachlan J.A., Schwartz J., Skakkebaek N., Soto A.M., Swan S., Walker C., Teresa K. Woodruff T.K., Woodruff T.J., Giudice L.C., Guillette L.J. (2008). Female reproductive disorders: the roles of endocrine-disrupting compounds and developpmental timing. Fertil Steril. 90 , 911-940.

Danyi, S., Brose, F., Brasseur, C., Schneider, Y.-J., Larondelle, Y., Pussemier, L., Robbens J., De Saeger S., Maghuin-Rogister G., Scippo M.L. (2009). Analysis of EU priority polyciclic aromatic hydrocarbons in food supplements using high performance liquid chromatography coupled to an ultraviolet, diode array or fluorescent detector. Analytica Chimica Acta 633 , pp. 293-299.

Dardenne, F., Nobels, I., De Coen, W., & Blust, R. (2007). Dose–response relationships and statistical performance of a battery of bacterial gene profiling assays. Appl. Microbiol Biotechnol , 223-234.

Dardenne, F., Nobels, I., De Coen, W., & Blust, R. (2008). Mixture toxicity and gene inductions: can we predict the outcome? Environmental Toxicology and Chemistry, Vol. 27, No. 3, , 509-518.

De Gryse P. and Berthot C., (2009), "Compléments alimentaires : Législation et commentaire concernant la notification", note published by the Federal Public Service (SPF SPSCAE), 19p.

Dialogue et Santé, (2010), "Over the Counter: les allégations de santé", in Dialogue et Santé, n°267, p. 34.

Diana Di Mavungu J, Monbaliu S, Scippo ML, Maghuin-Rogister G, Schneider YJ, Larondelle Y, Callebaut A, Robbens I, Van Peteghem C and De Saeger S (2009) LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment. 26:885-895.

Dolan P., Cookson R., Ferguson B. (1999) Effect of discussion and deliberation on the public's views of priority setting in health care: focus group study. BMJ Vol. 318 (3 April), pp. 916-919.

Durr D., Stieger, B., Kullack-Ublick, G., Rentsch, K., Steinert, H., Meier, P., et al. (2000). St John's Wort induces intestinal P-Glycoprotein/MDR1 and intestinal and hepatic CYP3A4. Clinical pharmacology and therapeutics 68 (6) , 598-604.

EFSA (2005). Opinion of the Scientific Committee on a request from EFSA related to A Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and Carcinogenic. The EFSA Journal (2005) 282, 1-31

EFSA (2008). Polycyclic aromatic hydrocarbons in food. Scientific opinion of the Panel on Contaminants in the Food Chain. (Question No EFSA-Q-2007-136) http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902034842.htm

EFSA (2009). EFSA Scientific Committee; Guidance on safety assessment of botanicals and botanical préparations intended for use as ingredients in food supplements, on request of EFSA. EFSA Journal 2009; 7(9):1249. [19 pp.]. doi:10.2093/j.efsa.2009.1249. Available online: www.efsa.europa.eu

EFSA (2009). Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on cadmium in food. The EFSA Journal (2009) 980, 1-139.

EFSA (2010). EFSA's Modus Operandi for Article 13 (3) Health Claims of Regulation (EC) No 1924/2006. www.efsa.europa.eu/en/ndaclaims13/docs/art13modusoperandi.pdf (consulted on 15/01/2011).

EFSA (2010). Scientific Opinion on Lead in food. EFSA Journal 2010; 8(4):1570 www.efsa.europa.eu/en/scdocs/1570.htm.

EFSA, 2006. Opinion of the Scientific Panel on contaminants in the food chain [CONTAM] related to ochratoxin A in food. The EFSA Journal (2006) 365, 1 – 56. http://www.efsa.europa.eu/en/scdocs/doc/365.pdf

EFSA. (2004). Discussion paper on "botanicals and botanical preparations widely used as food supplements and related products: coherent and comprehensive risk assessment and consumer information approaches". Brussels.

Europa (2010). Food: Commission reviews the progressive adoption of the list of permitted health claims. http://europa.eu/rapid/pressReleasesAction.do?reference=IP/10/1176&format=HTML&aged=1&language=EN&guiLanguage=en (consulted on 15/01/2011).

European Botanical Forum, "An introduction to the role, safety and benefits of botanical food supplements", 22p. (http://www.botanicalforum.eu/uploads/ebf_factsheets.pdf)

European Commission (2000). Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. OJ L 109, 6.5.2000, p. 29–42

European Commission (2001) Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use. OJ L 311, 28.11.2001, p. 67–128

European Commission (2002) Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food suppléments. **OJ L 183, 12.7.2002, p. 51–57**

European Commission (2003). Règlement (CE) N° 2065/2003 du Parlement Européen et du Conseil du 10 novembre 2003 relatif aux arômes de fumée utilisés ou destinés à être utilisés dans ou sur les denrées alimentaires. JO L309 du 26.11.2003, p. 1.

European Commission (2005). COMMISSION RECOMMENDATION of 4 February 2005 on the further investigation into the levels of polycyclic aromatic hydrocarbons in certain foods (2005/108/EC)

European Commission (2006). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. OJ L 364, 20.12.2006, p. 5–24

European Commission (2006). Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. OJ L 404, 30.12.2006, p. 9–25.

European Commission (2006). Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods. OJ L 404, 30.12.2006, p. 26–38

European Commission (2010). Draft amending the Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards to polycyclic aromatic hydrocarbons. C(2009) SANCO/10616/2009/rev. 3, 18/10/2010.

FAFSC (2009). Advice 36-2009 of the Scientific Committee of the FASFC on the estimation of dietary intake of lead by the Belgian population.

FASFC (2009). Advice 29-2009 of the Scientific Committee of the FASFC on emrging chemical risks. A case study : endocrines disruptors.

FASFC (2009). Advice 35-2009 of the Scientific Committee of the FASFC on the estimation ofthe dietary intake of cadmium by the Belgian population.

Focant JF, Eppe G, Scippo ML, Massart AC, Pirard C, Maghuin-Rogister G, De Pauw E. (2005) Comprehensive two-dimensional gas chromatography with isotope dilution time-of-flight mass spectrometry for the measurement of dioxins and polychlorinated biphenyls in foodstuffs - Comparison with other methods. *J Chromatogr A*. **1086** : 45-60

FSA (2003). Committee on Toxicity of Chemicals in Food,Consumer Products and the Environment: Phytoestrogens and Health. COT Report.

Gago J.M. (2004) Science policy for risk governance. European Molecular Biology Association reports, 5 pp. 4-6.

Gaigner C. and Hebel P., (2005), "Qui sont les consommateurs de compléments alimentaires ?", présentation au 2e colloque international de l'IREMAS, Paris, 20-21/10/2005, 16p.

Ganzera M., Zhao J., Khan I.A. Hypericum perforatum-Chemical profiling and quantitative results of St. John's Wort products by an improved high-performance liquid chromatography method. J. Pharm. Sci., 2001, **91**, 623-630.

Gonzales F.G. and Gonzales-Castañeda C. (2009) The Methyltetrahydro-β-Carbolines in Maca (*Lepidium meyenii*). Evid Based Complement Alternat Med. 6(3): 315–316

Gordon, R., Becker, D., & Rader, D. (2009). Reduced Efficacity of Rosuvastatin by St John's wort. The American Journal of Medicine.

Gradin, K., Whitelaw, M., Toftgaard, R., Poellinger, L., & Berghard, A. (1994). A tyrosine kinase-dependent pathway regulates ligand-dependent activation of the dioxin receptor in human keratinocytes. Journal of Biological Chemistry 269(38) , 23800-23807.

Helsby, N., Chipman, J., Gescher, A., & Kerr, D. (1998). Inhibition of mouse and human CYP 1A-and 2E1-dependent substrate metabolism by the isoflavonoids genistein and equol. Food and Chemical Toxicology 36(5) , 375-382.

Hukkanen, J., Lassila, A., Paivarinta, K., Valanne, S., Sarpo, S., Hakkola, J., et al. (2000). Induction and regulation of xenobiotic-metabolizing cytochrome P450s in the human A549 lung adenocarcinoma cell line. Em J Respir Cell Mol Biol 22 (3) , 360-366.

IPCS (2009a) Principles and methods for the risk assessment of chemicals in food. (Environmental health criteria ; 240). http://whqlibdoc.who.int/publications/2009/9789241572392_eng.pdf

IPCS (2009b) Principles for modelling dose–response for the risk assessmentof chemicals. Geneva, World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria, No. 239; http://whqlibdoc.who.int/publications/2009/9789241572392_eng.pdf).

ISO 9167-1-1992; Rapeseed -- Determination of glucosinolates content -- Part 1: Method using high-performance liquid chromatography.

Izzo, A., & Ernst, E. (2009). Interactions between herbal merdicines and prescribed drugs, an updated systematic review. Drugs - 69 (13) , 1777-1798.

Jacobs MN, Covaci A, Gheorghe A, Schepens P (2004) Distribution of OCPs, PCBs and PBDEs in dietary supplements based on fish oils rich in omega-3 fatty acids. Journal of Agricultural and Food Chemistry, 52: 1780-1788.

JECFA (2005). JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES. Sixty-fourth meeting. Rome, 8-17 February 2005. http://www.who.int/ipcs/food/jecfa/en/

JECFA (2011). Evaluation of certain food additives and contaminants (Seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 960, 2011

Kim, H.-J., Chun, H.-S., & Yang, R. (2000). Inhibition of benzo[a]pyrene-induced cytotoxicity and cytochrome P450 1A activity by dietary flavonoids in human liver cell model: structure-activity relationship. Biotechnology Letters 22 (24) , 1941-1946.

Klein, K., & King, A. (2007). Genistein genotoxicity: Critical considerations of *in vitro* exposure dose. Toxicology and Applied Pharmacology, 224 , 1 - 11.

Komorosky, B., Zhang, S., Cai, H., Hutzler, J., Frye, R., Tracy, T., et al. (2004). Induction and inhibition of cytochromes P450 by the St John's wort constituent hyperforin in human hepatocyte cultures. Drug Metab Dispos, 32 , 512-518.

Larondelle, Y., Schneider, J.-Y., Pussemier, L., Van Peteghem, C., & De Saeger, S. (2006). Interaction between mycotoxins and other food contaminants: a new safety concern, p.58. Belgium: Scientific Support Plan for a Sustainable Development Policy (SPSD II).

Latour B., (2001), "Nouvelles règles de la méthode scientifique", in Projet n°268, CERAS, Décembre 2001.

Le Vif/l'Express Extra, (2010), "Tout sur les médecines alternatives et complémentaires. Phytothérapie, homéopathie, acupuncture, sophrologie, kinesiologie, ...", 4e Cahier du Vif/l'Express n°46, 19 au 25 novembre 2010, 50p.

Liu F.F, Ang C.Y.W., Heinze T.M., Rankin J.D., Beger R.D., Freeman J.P., Lay Jr J.O. Evaluation of major active components in St. John's Wort dietary supplements by high-performance liquid chromatography with photodiode array detection and electrospray mass spectrometric confirmation. J. Chormatogr. B, 2000, **888**, 85-92.

Locatelli, C. (2008). Metals. Dans D. Barcelo, Comprehensive Analytical Chemistry - Vol 51 Food contaminants and residue analysis (pp. 571-598). Elsevier.

Loux F., (1990), "La médecine familiale dans la France rurale", in Anthropologie et sociétés, Vol. 14, n°1, pp. 83-92.

Mormont M., Muller M., Maghuin-Rogister G., Scippo M.L., Van der Heiden E., Ribonnet L., Larondelle Y, ves-Jacques Schneider Y.J., Callebaut A., Pussemier L., de Voghel S., Covaci A., Nobels I., Robens J., De Saeger S., Dianadimavungu J. Food Interactions : Effects On Health, Consumer Perception And Impact On Agro-Food Industries "Foodinter" (SD/AF/04A). Brussels : Belgian Science Policy 2009 –

66 p. (Research Programme Science for a Sustainable Development : Final Report Phase 1, available on the Belspo website).

Markowitz, J., Donovan, J., De Vane, C., Taylor, R., Ruan, Y., Wang, J., et al. (2003). Effect of St John's wort on drug metabolism by induction of cytochrome P45 3A4 enzyme. Journal of American Medical Association, vol 290, no.11 , 1500-1504.

Mc Comas K., (2004), "When even the 'best-laid' plans go wrong. Strategic risk communication for new and emerging risks", in European Molecular Biology Association reports, Vol. 5 (Special Issue), pp. 61-65.

Mc Grath, T., Sharma, R., & Hajaligol, M. (2001). An experimental investigation into the formation of polycyclic aromatic hydrocarbons (PAH) from pyrolysis of biomass materials. Fuel , 80: 1787-1797.

McClain, M., Wolz, E., Davidovich, A., Pfannkuch, F., Edwards, J., & Bausch, J. (2005). Acute, subchronic and chronic safety studies with genistein on rats. Food chem toxicol. 44 (1) , 56-80.

Moniteur Belge (1992). Arrêté royal du 3 mars 1992 concernant la mise dans le commerce de nutriments et de denrées alimentaires auxquelles des nutriments ont été ajoutés. MB du 15-04-1992, n°1992025086, page : 08467**.**

Moniteur Belge (1992). Arrêté royal du 8 JANVIER 1992 concernant l'étiquetage nutritionnel des denrées alimentaires. MB du 21-02-1992 numéro : 1992025029 page : 3657.

Moniteur Belge (1997). Arrêté royal du 29 AOUT 1997relatif à la fabrication et au commerce de denrées alimentaires composées ou contenant des plantes ou préparations de plantes. MB du 21-11-1997 numéro : 1997022673 page : 30898.

Moniteur Belge (1999) - Arrêté royal du 13 SEPTEMBRE 1999 relatif à l'étiquetage des denrées alimentaires préemballées. MB du 29-10-1999 numéro : 1999022945 page : 40908.

Moniteur Belge (2003). Arrêté ministériel du 21 MAI 2003 déterminant les formes chimiques et les critères de pureté pour les nutriments qui peuvent être utilisés pour des compléments alimentaires. MB du 08-07-2003 numéro : 2003022677 page : 36356

Moniteur Belge (2009a). Arrêté royal du 12 FEVRIER 2009 relatif à la fabrication et au commerce de compléments alimentaires contenant d'autres substances que des nutriments et des plantes ou des préparations de plantes. MB du 18-03-2009 numéro : 2008024355 page : 22828.

Moniteur Belge (2009b). Arrêté ministériel du 9 FEVRIER 2009 relatif à la fabrication et au commerce de compléments alimentaires contenant d'autres substances que des nutriments et des plantes ou des préparations de plantes. MB du18-03-2009 numéro : 2008024356 page : 22831

Montaut, S., Barillari, J., Iori, R., & Rollin, P. (2010). Glucoraphasatin: Chemistry, occurrence, and biological properties. Phytochem. (71) , 6.

Mormont M. (2002), "L'expertise en questions", in Collectif, "L'expertise en questions. Domestiquer l'incertitude dans la société du risque", Bruxelles, PIE-Peter Lang, pp.(in press)

Mormont M. and Mélard F., (2010), "The productivity of deliberative focus group in face of pesticides' risks", in Journal of Rural Studies – special issue "Research Practices and Sustainable Development", ed. Pergamon Press, Elsevier, 30p.

Mormont M. and Van Huylenbroeck G., (2001a), "A la recherche de la qualité. Analyses socioéconomiques des nouvelles filières agro-alimentaires", Liège, presses de l'Université de Liège, 200p.

Mormont M., (2001b), "Gouverner l'environnement", in Debuyst F et al., "Savoirs et jeux d'acteurs pour des développements durables", Louvain la neuve, Bruylant-Academia, pp. 221-236.

Mormont M., (2005), "Développement durable : construire des mondes communs", in Travail-Emploi-Formation, n° 5, pp. 11-25.

Mougenot C. & Mormont M., (2001), "Governing Biodiversity", in International Journal of Global Environmental Issues, 2, 1/2, pp. 78-99.

Moyad, M. (2010). Under-hyped and over-hyped drug dietary supplement interactions and issues. Urologic Nursing, vol 30 number 1 , 85-87.

Mueller SC, Majcher-Peszynska J, Mundkowski RG, Uehleke B, Klammt S, Sievers H, Lehnfeld R, Frank B, Thurow K, Kundt G, Drewelow B. Eur J Clin Pharmacol. 2009 Jan;65(1):81-7

Njumbe Ediage E., Diana Di Mavungu J., Scippo M.L., Schneider Y.J., Larondelle Y., Callebaut A., Robbens J., Van Peteghem C., De Saeger S.. Screening, Identification and Quantification of glucosinolates in black radish (*Raphanus sativus L. niger)* based dietary supplements using liquid chromatography coupled with a photodiode array and liquid chromatography – mass spectrometry. Journal of Chromatography A, 1218 (2011) 4395-4405.

Obach, R. (2000). Inhibition of Human Cytochrome P450 Enzymes byConstituents of St. John's Wort, an Herbal Preparation Used in the Treatment of Depression. The journal of pharmacology and experimental therapeutics , 88-95.

Oh, S., & Chung, K. (2006). Estrogenic activities of *Ginkgo biloba* extracts. Journal of steroid biochemistry & molecular biology100 , pp. 167-178.

Pang T. and Guindon E., (2004), "Globalization and risks to health", in European Molecular Biology Association reports, Vol. 5 (Special Issue), pp. 11-16.

Patisaul, H., & Jefferson, W. (2010). The pros and cons of phytoestrogens. Front. Neuroendocrinol. , doi:10.1016/j.yfrne.2010.03.003.

Polkowski, K., & Mazurek, A. (2000). Biological properties of genistein. A review of *in vitro* and *in vivo* data. Acta Pol Pharm. 57 (2) , 135-155.

Renn O. and Klinke A. (2004), "Systemic risks: a new challenge for risk management", in European Molecular Biology Association reports, Vol. 5 (Special Issue), pp. 41-46.

Renn O., (2003), "Acrylamide: Lessons for Risk Management and Communication", in Journal of Health Communication, Vol. 8: 5, pp. 435-441.

Ribonnet, L., Garsou, S., & Pussemier, L. (2007). Determination of realistic concentrations for studying toxic effects of food chemical contaminants at the gastro-

intestinal level. Platform for scientific concertation: Food safety - Toward a safer food supply in Europe , 283-301. SPSD II.

Ribonnet, L., Van der Heiden , E., Nobels, I., Chaumont, A., Remacle, A.-S., De Saeger, S., Schneider, Y.-J., Scippo, M.-L., Blust, R., Pussemier, L. and Larondelle, Y. (2011). Potential of an *in vitro* toolbox combined with exposure data as a first step for the risk assessment of dietary chemical contaminants. In press in Food Additives and Contaminants.

Ropeik D. and Slovic P., (2003), "Risk in perspective. Risk communication : a neglected tool in protecting public health", publication of the Harvard Center for Risk Analysis, Vol. 11, Issue 2, 4p.

Ropeik D., (2004), "The consequences of fear", in European Molecular Biology Association reports, Vol. 5 (Special Issue), pp. 56-60.

Rosin, A. (2009). The Long-term Consequences of Exposure to Lead. Isr Med Assoc J. 11:689-94.

Satarug, S., Garrett, S., Sens, M., & Sens, D. (2010). Cadmium, environmental exposure, and health outcomes. Environmental Health Perspectives , Vol 118, 182-190.

Satarug, S., Haswell-Elkins, M., & Moore, M. (2000). Safe levels of cadmium intake to prevent renal toxicity in human subjects. Br J Nutr 84(6). , 791–802.

SCF. (2002). Opinion of the Scientific Committee on Food on the risks to human health of Polycyclic Aromatic Hydrocarbons in food. SCF/CS/CNTM/PAF/29/Final.

Scheer D., Gold S., Benighaus C., Benighaus L., Ortleb J., Renn O., 2010, "Communication of Risk and Hazard from the Angle of Different Stakeholders", Federal Institute for Risk Assessment Press Office (BfR-Wissenschaft), Berlin, 149p.

Schwarz, D., Kisselev, P., & Roots, I. (2003). St. John's Wort Extracts and Some of Their Constituents Potently Inhibit Ultimate Carcinogen Formation from Benzo[a]pyrene-7,8-Dihydrodiol by Human CYP1A1. Cancer Research 63 , 8062-8068.

Scippo, M., Eppe, G., Saegerman, C., Scholl, G., De Pauw, E., Maghuin-Rogister, G., et al. (2008). Persistant Organochlorine Pollutants, Dioxins and Polychlorinated Biphenyls. Dans D. Barcelo, Comprehensive Analytical Chemistry - Vol 51 Food contaminants and residue analysis (pp. 457-506). Elsevier.

Sergent, T., Dupont, I., Van Der Heiden, E., Scippo, M.-L., Pussemier, L., Larondelle, Y., et al. (2009). CYP1A1 and CYP3A4 modulation by dietary flavonoids in human intestinal Caco-2 cells. Toxicology Letters 191 , 216-222.

Sergent, T., Ribonnet, L., Kolosova, A., Garsou, S., Schaut, A., De Saeger, S., et al. (2008). Molecular and cellular effects of food contaminants and secondary plant components and their plausible interactions at the intestinal level. Food and Chemical toxicology 46 , 813-841.

Shafritz L.B. and Roberts A., (1994), "The value of focus-group research in targeting communication strategies: an immunization case study", in Health Transition Review, Vol 4 n°1, pp. 81-102.

Shon, Y.-H., Park, S.-D., & Nam, K.-S. (2006). Effective Chemopreventive Activity of Genistein against Human Breast Cancer Cells. J Biochem Mol Biol 39 (4) pp.(4) , 448-451.

Stassart P. and Whatmore S., (2003), "Metabolizing risk: the assemblage of alternative meat network in Belgium", in Environment and Planning A, theme issue : Alternative agrifood networks :european perspectives, Bristol.

Sugimoto, K.-i., Ohmori, M., Tsuruoka, S., Nishiki, K., Kawaguchi, A., Harada, K.-i., et al. (2001). Different effects of St John's wort on the parmacokinetics of simvastatin and pravastatin. American Society for Clinical Pharmacology & Therapeutics , 518-524.

Tamakawa, K. (2008). Polycyclic Aromatic Hadrocarbons. in D. Barcelo, Comprehensive Analytical Chemistry - Vol 51 Food contaminants and residue analysis (pp. 599-651). Elsevier.

TNS Opinion & Social. (2010). Special eurobarometer 354: Food-related risks. EFSA.

Touvier M. & al., (2003), "Les forts consommateurs de compléments alimentaires en France. Résultats de l'enquête ECCA", in Cahiers de nutrition et de diététique, Vol.38 n°3, pp. 187-194.

Van Beek, T., & Montoro, P. (2009). Chemical analysis and quality control of *Ginkgo biloba* leaves, extracts and phytopharmaceuticals. Journal of Chromatography A 1216 (11) , 2002-2032.

Van Der Heiden, E., Bechoux, N., Muller, M., Sergent, T., Schneider, Y.-J., Larondelle, Y., Maghuin-Rogister, G., & Scippo, M.-L. (2009). Food flavonoid aryl hydrocarbon receptor-mediated agonistic/antagonistic/synergic activities in human and rat reporter gene assays. *Analytica Chimica Acta, 637*, 337-345.

Van der Sjaak, G. and Whyte S.R., (2003), "Popularité et scepticisme : opinions contrastées sur les médicaments", in Anthropologies et sociétés, vol. 27, n°2, pp.97-116.

Vromman V. , Waegeneers N., Cornelis C., De Boosere I., Van Holderbeke M., Vinkx C.; Smolders E.; A. Huyghebaert[f] A., Pussemier L. (2010). Dietary cadmium intake by the Belgian adult population. Food additives and contaminants 27 (12), p. 1665-1673.

Vuyslteke A, Collet E, Mormont M Van Huylenbroeck, (2003), "Effets de la normalisation sur la structure et l'organisation des entreprises dans l'agriculture", Gent, Academia Press, 177p.

Wang, Z., Gorski, J., Hammam, M., Huang, S., Lesko, L., & Hall, S. (2001). The effects of St John's wort (Hypericum perforatum) on human cytochrome P450 activity. Clinical pharmacology and therapeutics , 70(4): 317-26.

Wang, Z., Gorski, J., Hamman, M., Huang, S., Lesko, L., & Hall, S. (2001). The effet of St John's wort (Hypericum perforatum) on human cytochrome P450 activity. Clinical pharmacology and therapeutics 60 (4) , 317-326.

Weingart P., (2004), "Science in a political environment. The dynamics of research, risk perception and policy making", in European Molecular Biology Association reports, Vol. 5 (Special Issue), pp. 52-55.

WHO. (2000). Evaluation of certain food additives and contaminants, 55th Report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series 89.

Wiedemann P.M., Schütz H., Spangenberg A. (dir.), 2010, "Evaluation of Communication on the Differences between "Risk" and "Hazard"", Federal Institute for Risk Assessment Press Office (BfR-Wissenschaft), Berlin, 84p.

Wilkinson S. B.T., Rowe G. and Lambert N., (2004), "The risks of eating and drinking. Consumer perceptions and 'reality'", in European Molecular Biology Association reports, Vol. 5 (Special Issue), pp. 27-31.

Willard, S.T. and Frawley, L.S. (1998) Phytoestrogens have agonistic and combinatorial effects on estrogen-responsive gene expression in MCF-7 human breast cancer cells. *Endocrine* **8**, pp. 117–121.

Willemsen, P., M. L. Scippo, et al. (2002). "Use of specific bioluminescent cell lines for the detection of steroid hormone(anta)agonists in meat producing animals". Anal. Chim. Acta 473, 119-126.

Willemsen, P., M. L. Scippo, et al. (2004). "Use of reporter cell lines for detection of endocrine-disrupter activity." Anal Bioanal Chem 378(3): 655-63.

Willemsen, P., M. L. Scippo, et al. (2005). "Enhancement of steroid receptor-mediated transcription for the development of highly responsive bioassays." Anal Bioanal Chem 382(4): 894-905.

Williams B. & al., (2005), "Patient perspectives on multiple medications versus combined pills: a qualitative study", in Q J Med, n°98, pp. 885–893.

Wong L.P, (2008), "Focus group discussion: a tool for health and medical research", in Singapore Medical Journal, vol.49(3), pp. 256-261.

ANNEXES

Annex 1 : Copy of the publications

Danyi, S., Brose, F., Brasseur, C., Schneider, Y.J., Larondelle, Y., Pussemier, L., Robbens J., De Saeger S., Maghuin-Rogister G., Scippo M.L. (2009). Analysis of EU priority polyciclic aromatic hydrocarbons in food supplements using high performance liquid chromatography coupled to an ultraviolet, diode array or fluorescent detector. Analytica Chimica Acta 633 , pp. 293-299.

Diana Di Mavungu J., Monbaliu S., Scippo M.L., Maghuin-Rogister G., Schneider Y.J., Larondelle Y., Callebaut A., Robbens J., Van Peteghem C. and De Saeger S. (2009) LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment. 26:885-895.

Njumbe Ediage E., Diana Di Mavungu J., Scippo M.L., Schneider Y.J., Larondelle Y., Callebaut A., Robbens J., Van Peteghem C., De Saeger S.. Screening, Identification and Quantification of glucosinolates in black radish (*Raphanus sativus L. niger)* based dietary supplements using liquid chromatography coupled with a photodiode array and liquid chromatography – mass spectrometry. Journal of Chromatography A, 1218 (2011) 4395-4405.

Ribonnet L., Callebaut A., Nobels I., Scippo M.-L., Schneider Y.-J., De Saeger S., Pussemier L. and Larondelle Y. (2011). Modulation of CYP1A1 activity by a *Ginkgo biloba* extract in the human intestinal Caco-2 cells. Toxicology Letters 202: 193-202.

Annex 2 : Report of the sociological study (full length version)

Annex 3 : Program of the Foodinter Workshop, Tervuren, March 23, 2011

ANNEXES 2 & 3 ARE AVAILABLE ON OUR WEBSITE :

http://www.belspo.be/belspo/ssd/science/pr_agrofood_en.stm