Faculty of Pharmaceutical Sciences

# New and Selective Methods for the Detection of Phytoestrogens in Biological Matrices

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 1H and 13C NMR | proton and carbon nuclear magnetic resonance |
| 8-PN | 8-prenylnaringenin |
| Å | Ångström |
| ABTS | 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt |
| Ag-Ab | antigen-antibody |
| amu | atomic mass unit |
| AP | alkaline phosphatase |
| APCI | atmospheric pressure chemical ionization |
| APPI | atmospheric pressure photo-ionization |
| AT | Agilent Technologies |
| $B_0$ | absorbance in wells without presence of a competitor (= maximal binding of antibody) |
| CID | collision induced ionization |
| COUM | coumestrol |
| CVD | cardiovascular disease |
| DAID | daidzein |
| DCC | dicyclohexylcarbodiimide |
| DGAT | diglyceride acyltransferase |
| DHD | dihydrodaidzein |
| DMEM | dulbecco's modified eagle medium |
| DMF | dimethylformamide |
| DMSO | dimethyl sulfoxide |
| E2 | 17β-estradiol |
| EGF | epidermal growth factor |

| | |
|---|---|
| ELISA | enzyme-linked immunosorbent assay |
| END | enterodiol |
| ENL | enterolactone |
| EQ | equol |
| ER | estrogen receptor |
| ESI | electrospray ionization |
| FBS | fetal bovine serum |
| FIA | flow injection analysis |
| FORM | formononetin |
| FPLC | fast protein liquid chromatography |
| GEN | genistein |
| GC | gas chromatography |
| GLYC | glycitein |
| HAT | hypoxanthine aminopterine thymidine |
| HDL | high density lipoprotein |
| HMPA | hexamethylphosphoramide |
| HPLC | high performance liquid chromatography |
| HPRT | hypoxanthine phosphoribosyltransferase |
| HRT | hormone replacement therapy |
| $IC_{50}$ | concentration of competitor that gives 50% inhibition |
| ICC | immunocytochemistry |
| id | internal diameter |
| Ig | immunoglobulin |
| IP | immunoprecipitation |

| | |
|---|---|
| IQ | methylimidazol[4,5-f]quinoline |
| IS | internal standard |
| IX | isoxanthohumol |
| (k)Da | kilo Dalton |
| $\lambda_{max}$ | maximum wavelength |
| LC | liquid chromatography |
| LDL | low density lipoprotein |
| LLE | liquid-liquid extraction |
| LOQ | limit of quantification |
| *m*/*z* | mass-to-charge ratio |
| mAB | monoclonal antibody |
| MAT | matairesinol |
| MHz | MegaHertz |
| MOPC | mineral oil plasmacytoma |
| MS | mass spectrometry |
| MSD | mass selective detector |
| MWCO | molecular weight cut off |
| NC | negative control |
| NHS | N-hydroxysuccinimide |
| NR | nuclear receptor |
| *O*-DMA | *O*-desmethylangolensin |
| OPD | *O*-phenylethyldiamine |
| pAB | polyclonal antibody |
| PBS | phosphate-buffered saline |

| | |
|---|---|
| PBS(-Tw) | phosphate-buffered saline with 0.05% Tween 20 |
| PEG | polyethylene glycol |
| *p*NPP | *para*-nitrophenylphosphate |
| QC | quality control |
| *r* | Pearson's correlation coefficient |
| $r^2$ | correlation coefficient |
| RIA | radioimmunoassay |
| RP | reversed phase |
| SDS | sodium dodecyl sulfate |
| SECO | secoisolariciresinol |
| SERM | selective estrogen modulator |
| SPE | solid-phase extraction |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TLC | thin layer chromatography |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| TR-FIA | time-resolved fluoroimmunoassay |
| TSP | thermospray |
| UV | ultraviolet |
| % v/v | volume fraction |
| % w/w | mass fraction |
| WHI | Women's Health Initiative |
| X(N) | xanthohumol |

# PREFACE

Over the last few decades, several clinical trials have been conducted to clarify the possible benefits of phytoestrogens on different aspects of clinical health. The major hampering points in those studies are the lack of very well-defined test products and undefined molecular working mechanisms, which may lead to inconclusive and mixed results. The latter is often caused by inter-individual differences in metabolism and bioavailability. Nevertheless, distinct conclusions on the use of phytoestrogens in a clinical environment might be drawn if their absorption, bioavailability and metabolism could be analyzed.

Since the beginning of the investigations for phytoestrogens and their metabolites in a large variety of biological matrices, from crude plant materials over dietary supplements to biological fluids, several analytical techniques for their determination and quantification have been reported. These methods can be divided, based upon separation technology, into chromatographic and non-chromatographic methods. In the former group liquid (LC) and gas chromatography (GC) coupled to UV- and mass-detection ((tandem) MS) are the most reliable and commonly used ones. In the latter, immunoassays based on antibodies are the most popular and innovating ones.

Some important key points have to be kept in mind while choosing a correct analytical method. First of all, due to the fact that phytoestrogens sometimes have similarities in their structure, and the human metabolism can generate a wide range of metabolites, the chosen method has to be very specific. Next, when they are detected in biological fluids like urine or serum, their concentrations can be extremely low, even in the nanomolar region. Therefore, great sensitivity is another requirement. And, last but not least, when a large number of samples are generated in clinical studies, it is quite practical that the sample preparation and the method are as simple as possible, both for time and cost reduction.

# CHAPTER 1

## General introduction

*PHYTOESTROGENS FROM HOPS, SOY AND FLAX IN BIOLOGICAL MATRICES*

# 1. PHYTOESTROGENS

## *1.1 History and classes*

Plant-derived food products can form a source of phytoestrogens in the Western diet, but not all classes of phytoestrogens are consumed in the same amount or have the same potency, which has direct implications on their biological effects and their prevalence in biological fluids [1]. Therefore, it is important to draw attention to those phytoestrogens that are commonly consumed or are ingested through dietary supplements, *e.g.*, for the relief of menopausal symptoms. Phytoestrogens are secondary metabolites, typically phenolic compounds, synthesized by a plant, which may act as potent antioxidants [2]. In the mid-1920s, plant substances were already reported to cause estrus in animals [3]. Due to their aromatic conjugated structure phytoestrogens can serve as a protection mechanism against external influences like UV-radiation, micro-organisms or predators (*e.g.*, the outbreak of infertility among sheep grazing from red clover in Western Australia in the late 1940s [4]).

Phytoestrogens mimic the endogenous mammalian 17β-estradiol ($E_2$), both structurally and functionally. Classically, they are defined to exert estrogenic effects on the central nervous system, induce estrus and stimulate the growth of the genital tract of female animals. Moreover, they can interfere with endogenous estrogen signaling and associated processes. The ability to act both estrogenically as estrogen agonists and antiestrogenically as antagonists leads often to their classification as selective estrogen modulators (SERMs) [5]. Consequently, the use of several plants in traditional medicine, may often be ascribed to those diverse biological properties. The first report by Loewe *et al.* [6] of the estrogenic properties of plant extracts appeared in 1927, and half a century later the list of plants exhibiting estrogenic activity reached already several hundred [7]. Equol, an isoflavan from soy (*Glycine max* (L.) Merr.), was the first detected phytoestrogen in human urine, by Axelson *et al.* [8] and became one of the most studied phytoestrogens. Through

epidemiological studies and dietary surveys, the ingestion of phytoestrogens was linked to health-beneficial effects on Western diseases like cardiovascular disorders and breast and prostate cancer [2, 9]. Since the announcement of the results of the Women's Health Initiative (WHI) study [10] and Million Women Study [11], concerning the negative effects of hormone replacement therapy (HRT), phytoestrogens have been in the spotlights as an interesting alternative for the relief of menopausal symptoms.

Based on their chemical structures, the most consumed phenolic phytoestrogens can be classified into four main groups, the flavonoids, isoflavonoids, lignans and stilbenes (Figure 1.1). The first two groups are both derived from the heterocyclic hydrocarbon chroman, with substitution on C2 or C3 with a phenyl-group, for the flavonoids and isoflavonoids respectively [1, 4]. Further modifications of the basic structure of flavonoids results in three subgroups, *i.e.* flavones, flavanones and chalcones. The isoflavones and coumestans are the main players in the group of the isoflavonoids. The lignans differ in structure from the others with their dibenzyl-butane backbone. The last small group of the stilbenes, has resveratrol from grapevines (*Vitis vinifera* L.) as the most known representative but will not be further discussed in this thesis.

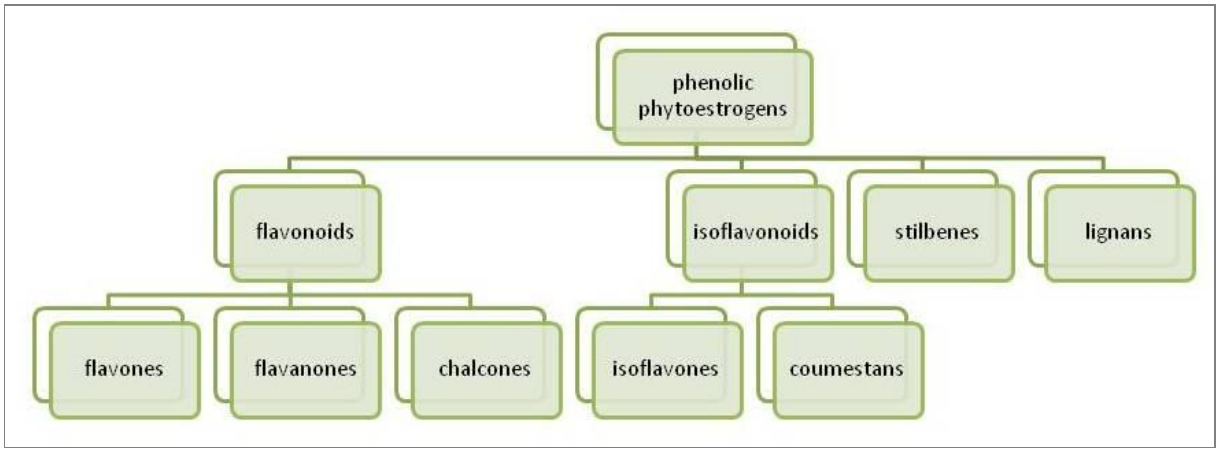



**Figure 1.1:** Classification of dietary phytoestrogens.

The aromatic ring and a hydroxyl group are important features for the binding of any chemical with the estrogen receptors. Additionally, the steric and hydrophobic properties, size and branching of the alkyl group and its location on the phenolic ring, will play an important role in their binding capacity. The structural similarity of estrogenic flavonoids to $E_2$ lies in their composition of a planar ring system that includes a *p*-hydroxy-substituted aromatic ring that is approximately 12 Å away from a second in-plane hydroxyl group [12] (Figure 1.2). Generally, phytoestrogens have a narrow structural-activity relationship, which is influenced by certain structural features or deviations and often metabolisation can play a crucial role.

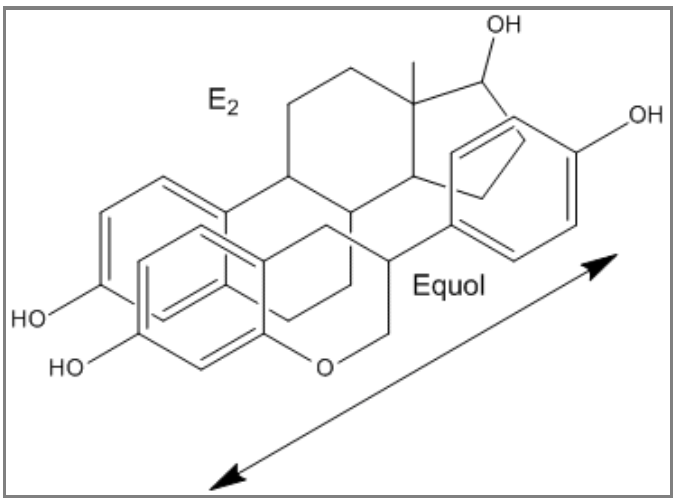



**Figure 1.2:** **Structural relationship between the endogenic 17β-estradiol and the flavan equol (arrow indicates the similar distance between the two hydroxyl-groups of both structures).**

## *1.2 Occurrence and sources*

- The family of the Leguminosae represents almost exclusively the **isoflavones**, which are also the most studied group of phytoestrogens [4]. Isoflavones are found in legumes and beans and side-products, but soybeans (*Glycine max* (L.) Merr.) are the main dietary source (1.2-2.4 mg/g dry weight) [13]. Nevertheless, isoflavone content of soy products widely varies, first of all due to different varieties, crop years and location [14] and secondly through processing like filtration, rinsing and extraction with alcohol [15].

Daidzein, genistein and glycitein are the major isoflavones and they occur in plants as β-D-glycosides daidzin and genistin and their respectively 4'-methyl ether derivatives formononetin and biochanin A (Figure 1.3). Despite the high stability of the glycosides during processing, once ingested, they are enzymatically hydrolyzed by the microflora to their corresponding aglycones. This biotransformation can result in the formation of dihydrodaidzein, *O*-desmethylangolensin (*O*-DMA) and finally equol from daidzein and dihydrogenistein, which is metabolized into 6'-hydroxy-*O*-desmethylangolensin, and the latter into 1,3,5-trihydroxybenzoic acid and finally *p*-ethyl-phenol, all originating from genistein [13, 16] (Figure 1.3). The flavan equol, which is a more potent phytoestrogen than its precursor daidzein, can be formed by only 30% of the humans, which contributes to the important inter-individual variability that is observed in studies [17].

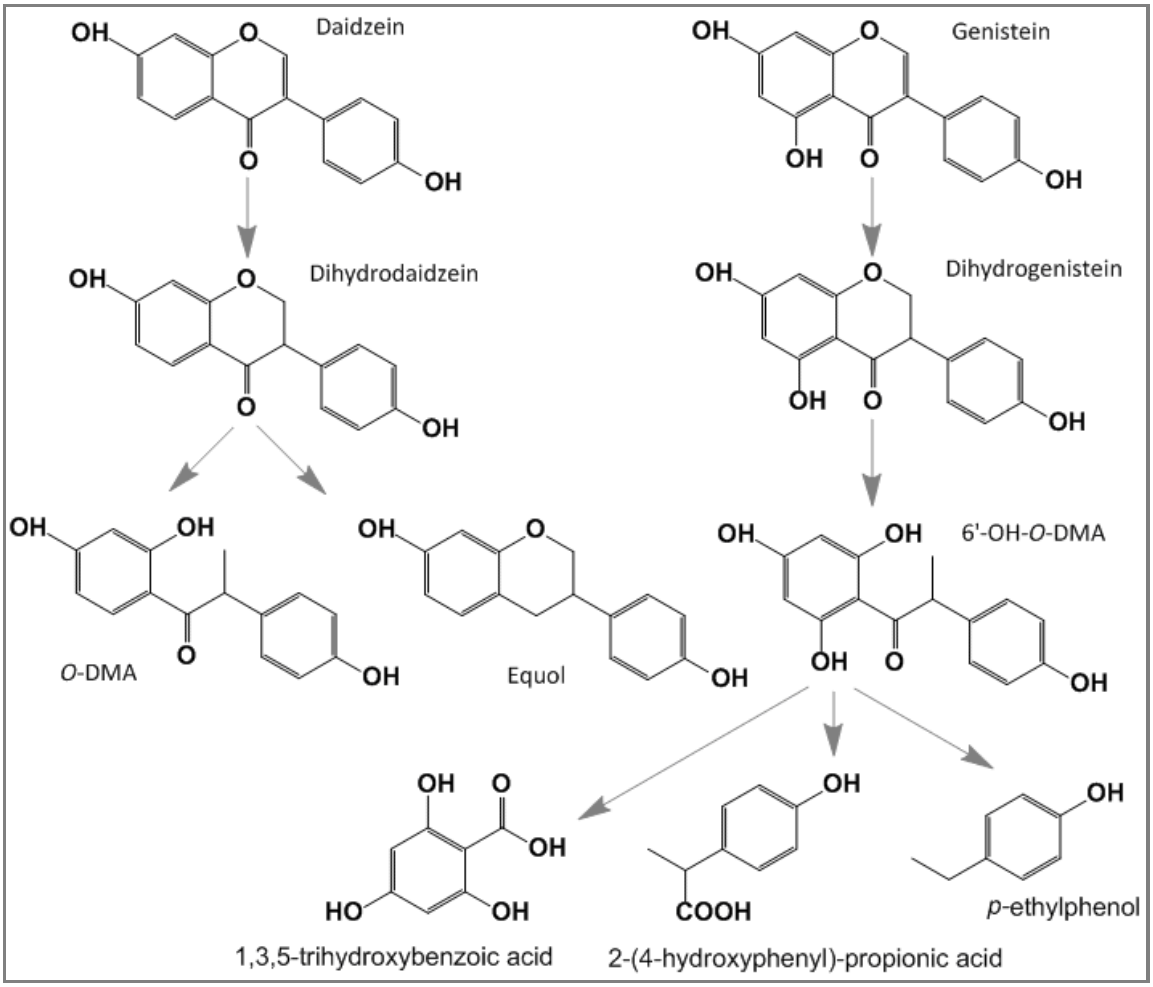



**Figure 1.3:** **Bacterial (gut) metabolism of the isoflavones daidzein and genistein.**

- The female inflorescences of the hop plant (*Humulus lupulus* L.), a perennial climbing vine in the Cannabaceae family, which are used in the brewing industry to add flavor and bitterness to the beer, are the dietary source of several **prenylated flavonoids** (with an average daily intake of 0.14 mg prenylflavonoids). More specific, the polyphenolic fraction of hops includes the flavanones 8-prenylnaringenin (8-PN), 6–prenylnaringenin (6-PN), isoxanthohumol (IX) and their prenylated chalcones desmethylxanthohumol and xanthohumol (XN) (Figure 1.4). The most abundant chalcone in hops (up to 1% w/w in dry cones) and concomitantly beer, is XN, which can be thermally isomerised into the proestrogen IX during the brewing process [18]. The latter is prone to metabolisation to the very potent phytoestrogen 8-PN, by liver enzymes [19] or intestinal gut bacteria. In parallel with equol, this last process is also depending on the right microbial community for the conversion [20] and can be of great influence on the biological effectiveness. Although the concentration of 8-PN in beer is quite low (less than 20 μg/L) [21], its occurrence has to be considered, given the fact that it is a very potent phytoestrogen and the possible metabolic conversion of the more abundant IX.

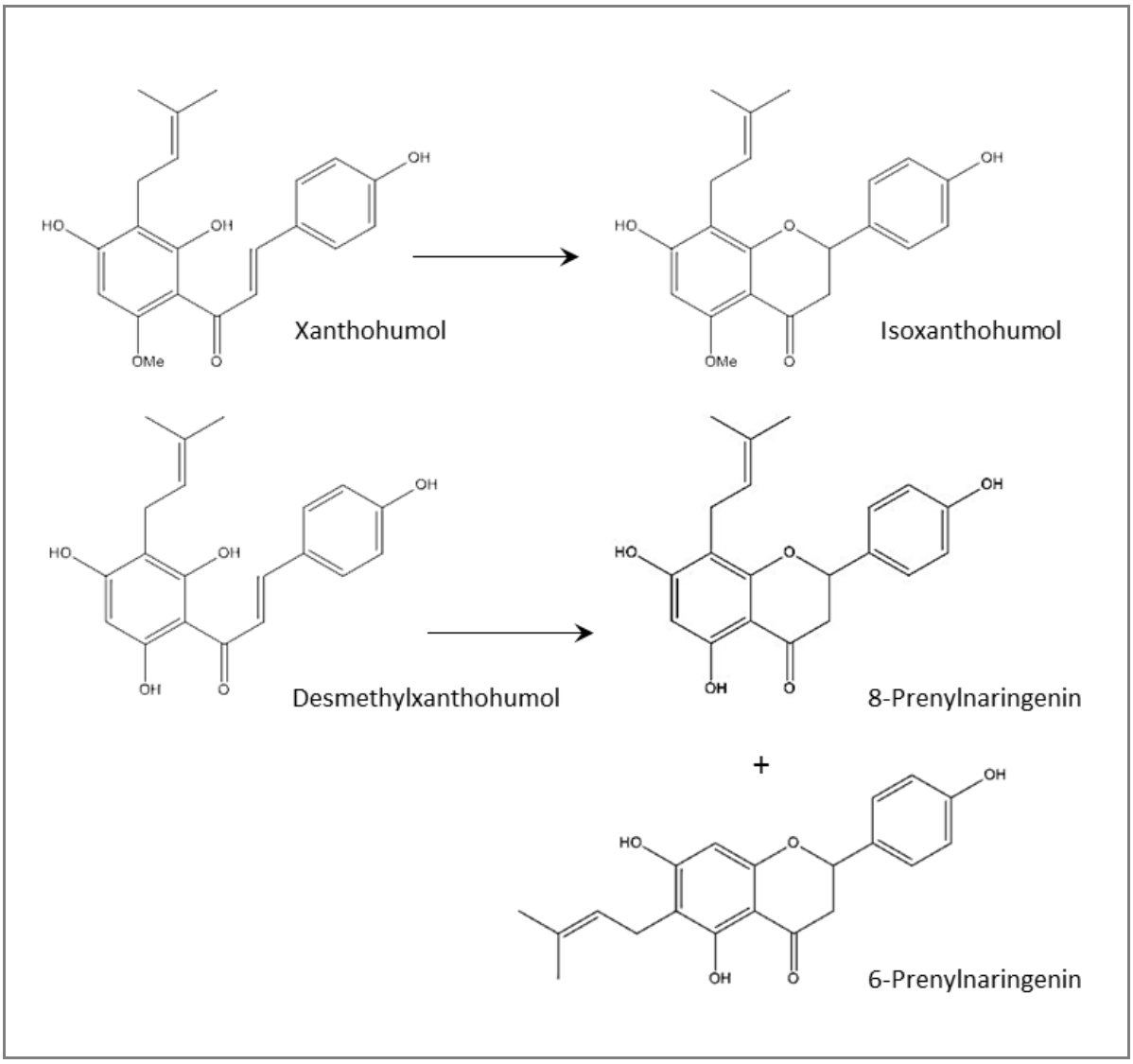



**Figure 1.4:** **Prenylated flavonoids from hops (*Humulus lupulus* L.). Thermal isomerization can give rise to isoxanthohumol from xanthohumol and 8-prenylnaringenin and 6-prenylnaringenin from desmethylxanthohumol.**

- **Lignans** are abundant in the most fibre-rich food sources like cereals, fruits and vegetables, with rye and flax seed (*Linum usitatissimum* L.) among the most important ones. Typically, flaxseed contains around 0.8 mg secoisolariciresinol per g dry weight [13] and this content increases after crushing and defatting [22]. The so-called mammalian lignans enterodiol and enterolactone, are the active phytoestrogens and are formed by microflora in the proximal colon, out of respectively, secoisolariciresinol and matairesinol, which occur as glycosides in the plant [23] (Figure 1.5). Enterolactone can

also be formed out of enterodiol. Also pinoresinol and lariciresinol, both precursors of secoisolariciresinol, as well as syringaresinol, can be metabolized to enterolactone [24]. Thus, many plant lignans from whole grain products can be converted to phytoestrogens.

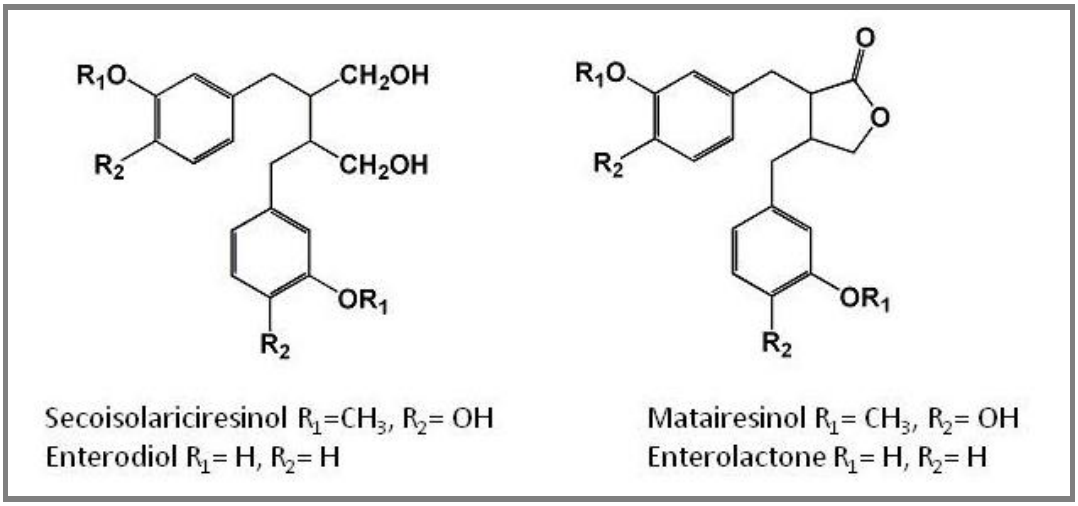



**Figure 1.5:** Mammalian lignans and their precursors.

- The **coumestans** coumestrol (Figure 1.6) and 4'-O-methylcoumestrol have as most significant sources sprouts of clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.), with a coumestrol content of 5.6 mg and 0.7 mg/g dry weight, respectively [25].

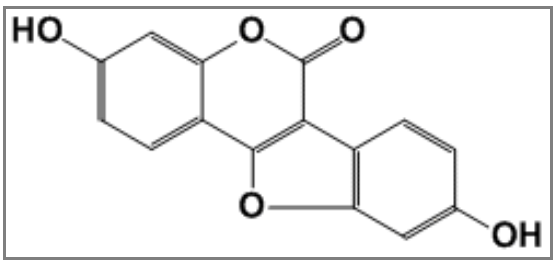



**Figure 1.6:** Coumestrol.

## *1.3 Oral bioavailability and disposition*

The assessment of bioavailability of phytoestrogens must be based on data from absorption, distribution, metabolism and excretion (ADME) and implies the extent and rate at which they become available in the general circulation. Generally, the oral bioavailability of isoflavones and lignans has already been well characterized in humans. In fact, a rather efficient absorption is observed after a single dose of aglycone equivalents of the isoflavones genistein and daidzein, with peak plasma concentrations appearing after 5.2 h and 6.6 h, respectively [26]. The same for enterolignans which appear in the plasma 9-10 h after the ingestion of purified secoisolariciresinol diglucoside [27]. In contrast, there are very few data on the bioavailability of prenylflavonoids. The ingestion of an oral single dose of 8-PN results in a first serum peak 1.0-1.5 h and a second peak after 7-8 h [28, 29]. Unfortunately, the hop-derived XN was suspected to have a very poor systemic bioavailability according to results from experiments with Caco-2 monolayers [30], and, in fact this was later confirmed by Bolca *et al.* in a human *in vivo* trial [31].

Flavonoids, isoflavonoids and lignans mostly occur as inactive compounds in the plants and food products, mainly as glycosidic conjugates, except for the prenylated hopflavonoids which are already in their aglycone form [1]. The bioavailability depends on complex enzymatic conversions in the gastrointestinal tract and starts with the formation of the active form of the compounds through hydrolysis by membrane-associated β-glucosidases in the small intestine or members of the colonic microflora [32-34]. This deglycosylation is considered crucial, as proven by studies with human intestinal epithelial cell monolayers, where the aglycones are transported and their glycosides not [35, 36]. Moreover, glucosides of isoflavones have not been identified in plasma either [36]. Absorption of the formed aglycones takes already place by the enterocytes of the small intestine or lower, in the large intestine. Nevertheless, before this absorption takes place, intestinal bacteria can already accomplish some metabolisation, *e.g.* formation of enterolignans enterodiol and enterolactone from the most important precursors (iso)lariciresinol, pinoresinol and

matairesinol. Moreover, this metabolism appears to be a requirement for absorption of the former compounds, as concentrations of the parent compounds in blood are negligible [23].

Then, via the portal vein, the absorbed compounds get distributed throughout the body and get conjugated with glucuronic and sulfuric acid by phase II enzymes (UDP-glucuronosyltransferases and sulfotransferases) in the liver, a process that can also occur in the intestinal epithelium. Phase II conjugation, the main metabolism for isoflavones and lignans results preferentially in conjugation at position 7 of the molecule and gives primarily monoglucuronides [37, 38]. Also for XN (*in vitro*) [39] and IX and 8-PN, not phase I metabolism, but preferentially extensive phase II conjugation is observed [29, 40, 41]. The conjugated aglycones are then excreted either through urine or through bile, which in the last case implies their enterohepatic circulation. This recirculation exposes the conjugated compounds again to deconjugating microbial enzymes and promotes the re-uptake or further metabolism and degradation in the lower intestine or even fecal excretion [13]. For example, equol peaks in plasma can be observed even 36 h post ingestion [34] and for enterolactone the peak plasma and urinary concentrations were measured at 24 h and between 25 h - 36 h respectively.

The metabolic conversions that plant isoflavones, lignans and prenylflavonoids undergo, can result in hormone-like compounds with an altered biological activity (increased or decreased bioactivity). Furthermore, these transformations, which depend largely on the intestinal environmental conditions, show considerable individual variation due to, among others, different consumption patterns, gender, age, food matrix, early exposure, chemical composition and *e.g.* the administration of antibiotics, which may completely put a stop to any conversion [34]. Concerning the conversion of daidzein, secoisolariciresinol and IX, into, respectively, *O*-DMA and equol; enterodiol and enterolactone; and 8-PN, it is even possible to classify the individuals into, weak, moderate and strong phenotype producers [42]. This variability is highly reflected in the reports of urinary and plasma levels of phytoestrogens from most clinical studies with phytoestrogen-containing food or dietary supplements.

## *1.4 Biological activities*

Although some early work has been conducted on isoflavones, the last two decades, they received more widespread attention for their possible role in disease prevention. The lignans and prenylflavonoids have also been studied likewise, moreover because of the suggestion to use them in dietary supplements as a safer alternative for the classical though controversial hormone replacement therapy. Besides that, numerous studies were dedicated to the investigation of other physiological effects and to the elucidation of the mechanisms by which they might function. The prediction of the *in vivo* effects of phytoestrogens is a difficult task, as it depends on numerous factors like route of administration, bioavailability, half-life, dose and duration of intake, intrinsic estrogenic state and nonhormonal secondary mediated actions [43]. In fact, their wide and complex range of biological activities can roughly be divided into estrogenic and antiestrogenic effects and others, like various cancers and cardiovascular diseases [44].

As aforementioned, phytoestrogens, because of their **estrogenic and antiestrogenic actions** *in vitro* and *in vivo*, are often considered as selective estrogen receptor modulators or SERMs. Like endogenous estrogens, phytoestrogens can regulate processes in a wide variety of tissues, mostly by interacting with nuclear estrogen receptors (ER). Up till now, two isoforms of ER have been identified, *i.e.* ERα and ERβ, which differ in the N-terminal ligand independent transactivation domain and ligand binding domain at the C-terminus, as well as in tissue distributions and relative binding affinities [45]. Both ERα and ERβ are expressed in utero-ovarian, breast and vascular tissue, ERβ mainly in brain, bone, lung tissue and prostate [46].

Regarding to the estrogenic potency, which can be deduced from *in vitro* bioassays, 8-PN, still takes first place of all phytoestrogens, relative to the naturally occurring 17β-estradiol [47, 48]. 8-PN is considered to be a pure ERα-agonist, exhibiting an estrogenic activity profile comparable to estrone, and is 70 times less potent than 17β-estradiol [48].

Furthermore, it exerts a bone tissue-specific estrogenic effect, with minimal trophic effects on uterus and endometrium [49]. Therefore, 8-PN is suggested as a potential compound for HRT in menopausal women. Nevertheless, more *in vivo* trials with target groups are required, although preliminary results from a randomized, placebo-controlled trial with a hop extract by Heyerick *et al.* [50] showed a promising outcome on the relief of menopausal discomforts. Next, with decreasing estrogenic potency and in following order come coumestrol, genistein, equol and daidzein which all are partially ERβ selective agonists [46, 47]. Both enterolignans enterodiol and enterolactone have even less estrogenic activity, the latter 10-fold higher than the former [51]. Although XN and IX do not possess any estrogenic activity themselves, due to bio-activation they can be categorized as pro-estrogens [19, 52]. At high plasma levels, the antiestrogenic effects of phytoestrogens may be provoked by competition with endogenous estrogens for binding with the ER, which can result in prevention of estrogen-stimulated cancer growth [53]. At micromolar concentrations, genistein, daidzein, equol and enterolactone showed estrogenic effects *in vitro* [54], but genistein, on the other hand, at higher concentrations also inhibited cell growth of human cell lines [55]. This last effect appeared not to be solely ER-dependent, as it also occurred in the ER-negative cell line. XN has been described to have an antiestrogenic activity by Gerhauser *et al.* [56], which was later refuted by Overk *et al.* [57]; however, both studies were conducted using a different methodological approach.

In the Western world, breast, prostate and colorectal **cancer** still belong to the most leading causes of mortality [58]. A large body of epidemiological studies showed that consumption of food rich in isoflavones and lignans may contribute to the lower incidence rates of former diseases in Asian countries [59, 60]. Their protective effects may be due to the lowering of circulating levels of unconjugated sex hormone, as soy isoflavonoids and lignans were shown to increase the levels of sex hormone binding globulin (SHBG) [61]. In women, newly diagnosed with breast cancer, a high excretion of both equol and enterolactone was associated with reduced breast cancer risk, when compared with matched controls [62]. It has been noted that early exposure to genistein, with its dual activity on cancer cells and more effective in inhibiting growth of non-neoplastic human

mammary cell lines than mammary cancer cells, may be important for breast cancer chemoprevention [63]. This narrow balance between risk or prevention, presumably depending on dietary intake levels, is also observed with coumestrol and enterolactone [64]. A significant decrease of the urinary excretion of the genotoxic metabolites of estradiol and estrone, *i.e.* 16α-hydroxyestrogens and 4-hydroxyestrogens, has been correlated with higher ingestion of soy and flax-derived phytoestrogens. The ratio 2-hydroxyestrogens, which are known to exert protective effects and are negatively correlated with the level of SHBG, to 16α-hydroxyestrogens is considered to be an important biomarker. Although, the evidence for possible preventive effects of phytoestrogens is sometimes conflicting. Importantly, the effects seem to be depending on the hormonal status of the women (pre- or postmenopausal).

Then, concerning the possible cancer-related activities of hop-derived prenylated flavonoids, major attention has been focused on XN. The latter seems to inhibit *in vitro* the initiation, promotion and progression stages of carcinogenesis and therefore, appears as broad-spectrum chemopreventive agent [65]. Xanthohumol works on different pathways that may be of concern in the development of cancer, first, already at low micromolar concentration, by the inhibition of metabolic activation of procarcinogens, a process catalyzed by cytochrome P450 enzymes [66, 67]. This mechanism was demonstrated by Miranda *et al.* [66] for XN, but also for IX and 8-PN, which are potent inhibitors of the metabolic activation of the heterocyclic amine, 2-amino-3-methylimidazol[4,5-*f*]quinoline (IQ). Secondly, several research groups proved that XN is capable of inducing the detoxification enzyme (phase II enzyme), quinone reductase (QR), which is a marker of chemoprevention [56, 68, 69]. Finally, XN may also prevent the growth of tumors at an early stage, for instance by the induction of apoptosis [70] or the inhibition of inflammatory signals by inhibition of cyclooxygenase (COX) enzymes [56] and may, indirectly influence, by the inhibition of NO (nitric oxide) production, the induction of angiogenesis [71]. On the other hand, little is known concerning the possible chemopreventive effects or even adverse effects of the full ERα agonist 8-prenylnaringenin, and its precursor isoxanthohumol. 8-PN showed similar growth-stimulatory effects as 17β-estradiol in the human MCF-7 breast

cancer cell line [72]. Nevertheless, recently, Brunelli and co-workers suggested for 8-PN inhibitory effects on epidermal growth factor (EGF)-triggered pathways [73] and dose-dependent apoptosis in breast cancer cells [74]. Other mechanisms, such as inhibition of aromatase activity [75, 76] and anti-angiogenic effects [77] have also been proposed. Bolca *et al.* [78], in their bioavailability study with hop prenylflavonoids, noted negligible hop-derived $E_2\alpha$- and $E_2\beta$-equivalents in human breast tissue (compared to the $E_2$ tissue exposure) after a 5-day oral treatment period with hop-derived prenylflavonoids, which are unlikely to provoke adverse effects. More studies with longer-term administration will be required to rigorously evaluate possible effects and draw concise conclusions about the safety of the use of hop-derived food supplements.

Another important focus has been on the possible health effects of phytoestrogens in **cardiovascular diseases** (CVD), a major death cause for both sexes, but especially for postmenopausal women, who suffer from an increasing risk due to a deficiency of gonadal steroid production. Apart from the direct influence on the vascular system due to the loss of endogenous estrogens, in general, postmenopausal women also show alterations in their lipoprotein profile. In fact, they mostly suffer from a decrease of the plasma concentrations of high density lipoprotein (HDL) and increasing plasma concentrations of low density lipoprotein (LDL) cholesterol [13]. The cardioprotective effect of higher estrogen levels or premenopausal hormone replacement therapy after menopause is already well established, but epidemiological data support that also the consumption of phytoestrogens can make a contribution [9]. Already for many years soy protein has been recognized for its hypocholesterolemic effect and a meta-analysis of 38 controlled clinical trials by Anderson *et al.* [79] has reported the reduction of total, LDL cholesterol and triglycerides by soy consumption. Although it must be emphasized that generally the effect on normocholesterolemic individuals is less or even not detectable [80]. Moreover, several clinical trials using isolated isoflavones have not shown a beneficial effect on serum lipids and, therefore, it is generally accepted that the protein matrix is necessary for the effectiveness of isoflavones on blood lipids [81-83]. The hypocholesterolemic effects may be acquired by an increased bile acid secretion and removal of LDL by hepatocytes as well as an

enhanced thyroid function [84]. Other mechanisms for the isoflavones, have been suggested, especially for genistein, which could acts as an inhibitor of tyrosine-specific protein kinases *in vitro*, and in this way may influence the process of coagulation [85]. However, in a clinical trial these results could not be observed after intake of soy supplements [86]. The antioxidant activity of soy isoflavones, *in vitro* and *in vivo*, provoked by a direct or indirect enhanrecement of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase enzymes, was reviewed by Kurzer and Xu [13] and affects the resistance of LDL to oxidation. The association between lignan intake and CVD has been studied to a far lesser extent.

A prospective large-scale study among Dutch women has reported on the relation between lignan intake and cardiovascular disease risk. The results did not support the overall protective effects of dietary intake at the habitually low level, with the exception of a small risk reduction for smokers with a higher lignan intake [87]. Moreover, no association between dietary intake of lignans and CVD could be found in another prospective study [88]. One study, albeit, reported lower risk of acute coronary events and CVD-related death in men [89]. In post-menopausal women a higher intake of lignan and isoflavonoid containing foods was associated with a more favorable waist to hip ratio, triglyceride levels, metabolic score and aortic stiffness [90, 91]. Generally, clear clinical endpoints concerning the possible risk reduction of CVD by lignans have not been established yet, according to Peterson *et al.* [92] mainly due to differences in dosages between intervention studies and habitual Western diet patterns.

The possibilities of applying coumestrol and prenylated flavonoids in CVD prevention have been scarcely explored. Their antioxidant properties will certainly be of importance in this health-related topic. For instance, Miranda *et al.* [93] and Stevens *et al.* [94] demonstrated that prenylated chalcones, especially XN, can inhibit oxidation of human LDL, at low micromolar concentrations. Furthermore, the inhibitory activity of XN against diacylglycerol acyltransferase (DGAT) can reduce the triglyceride synthesis and indirectly influence vascular health [95].

# 2. ANALYSIS OF PHYTOESTROGENS IN BIOLOGICAL MATRICES

## *2.1 Introduction*

The determination of phytoestrogens in biological samples, either qualitatively or quantitatively, requires an optimized procedure, mostly unique for the sample, the target compounds and the used analytical method. Nevertheless, the growing laboratory costs for both staff and materials, necessitate the development of simple, but efficient protocols, which is a very challenging task. This starts with a dedicated clean-up procedure of the sample, which depends on the origin of the sample, the target compound(s) and the used analytical method. In the very beginning, phytoestrogens were analyzed with the use of imprecise and insensitive techniques, such as thin layer and paper chromatography. Then, in the mid-1980s, the principal method for phytoestrogen analysis was gas chromatography coupled with mass spectrometry (GC-MS), a technique which is nowadays still being used, although it is very labor intensive [96]. More commonly applied, mainly because it requires less sample preparation and is a relatively rapid way to separate and quantify phytoestrogens, is high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. When more sensitivity is required, to measure concentrations of less than parts per million, the ultimate solution is HPLC coupled with (tandem) MS. The latter requires a less destructive sample preparation than for GC-MS, where conjugating groups must be removed prior to analysis. Nevertheless, the measurement stays dependent on the availability of internal standards for each analyte and the ability to produce molecular ions. Ultimately, there is a general and great demand for more rapid forms of analysis, and the non-chromatographic immunoassay techniques may offer a reasonable solution for this problem.

The second section of this general introduction will mainly discuss the analytical methods that were relevant for this study and were applied during the research, more specifically, liquid chromatography-mass spectrometry (LC-MS) and immunotechniques based on monoclonal antibodies (mAbs) such as an enzyme-linked immunosorbent assay

(ELISA), immunocytochemistry (ICC) and immunoprecipitation (IP) techniques. The present state of the art of the former techniques in the analysis of phytoestrogens will be outlined.

## *2.2 Sample preparation*

Generally, sample preparation for the analysis of phytoestrogens in food supplements is relatively simple compared to the one for biological samples. Phytoestrogens and their metabolites are present in biological samples such as urine and serum in parts per billion to parts per million concentrations, therefore, enrichment of the sample is in most cases necessary, also depending on the type of analysis that is being used. Before analysis there is also a need to separate them from the major constituents of these matrices. This isolation or extraction is not always 100% efficient and the addition of an internal standard (IS) is highly recommended to correct for unknown losses. The most commonly used internal standards range from deuterated ($^{2}H$) or carbon-13 ($^{13}C$) labeled stable isotopes or compounds that possess identical structural and chemical properties as the analytes of interest. The internal standards are naturally not present in the sample and have to be added in known concentrations. Correction is then done using the ratio of the analyte to the internal standard, measured during analysis. Nevertheless, no correction is made for losses before addition of the IS, during the hydrolysis of glucuronidated or sulfated ester forms of the phytoestrogens. Importantly, in biological fluids predominantly those conjugated forms are present, besides 1-5% of the total phytoestrogens that may be in the unconjugated form. Therefore, glycosides, glucuronidated or sulfated ester forms of suitable analogous compounds have also been used to correct for losses during extraction or incomplete hydrolysis [96, 97].

To convert conjugated forms of the phytoestrogens into their aglycones, an enzymatic method is commonly used, applying a mixed β-glucuronidase/sulfatase preparation from *Helix pomatia*. Because biological samples often contain organic substances like proteins and lipids or other suspended material, a filtration step at the beginning of the sample preparation or at time of collection can be required. Although, a centrifugation step can also

be used for that purpose. For the analysis of plasma samples, protein precipitation with acids or water-miscible organic solvents has been an attractive technique, due to several advantages such as its speed, simplicity of application and universality. Nevertheless, the sample can never get so clean as with liquid-liquid extraction (LLE) or solid phase extraction (SPE) and in the end may require longer run times due to LC or MS problems or cause matrix effects, which has made the use of this traditional technique less obvious. Furthermore, the availability of automated LLE or SPE extraction in 96-well plates has made them even more attractive. This automation is especially recommended for the routine analysis of large batches of clinical samples, when accurate, precise and rugged high-throughput sample preparation is needed. SPE is commonly performed with silica-based reversed phase C-18 cartridges, although newer materials like copolymers of N-vinyl-pyrrolidone and divinylbenzene, generating hydrophilic-lipophilic balanced reversed phase sorbents (trade named as Oasis HLB) start to gain popularity. LLE is performed using either diethyl ether or ethyl acetate. Even a combination of both SPE and LLE techniques can be used [96, 98].

### *2.3 Liquid chromatography – (tandem) mass spectrometry (LC-(MS/)MS)*

In contrast to the very labor-intensive gas chromatography (GC), which has been commonly used for many years in the analysis of phytoestrogens, because of its potential of high resolution, selectivity and sensitivity, liquid chromatography (LC) obviates the need for derivatization. All phytoestrogens and their metabolites contain at least one aromatic structure, and therefore are able to absorb UV-light (with a maximum wavelength ($\lambda_{max}$) ranging from 230 nm to 280 nm). Using a diode array detector (DAD), it is even possible to register the complete UV-spectrum of the molecule, eliminating the problems caused by differences in maximum UV absorption. Nevertheless, the concentrations of phytoestrogens and their metabolites in biological samples are generally too low to be measured with UV-detection alone, and the non-specificity of this detection method, has led to the coupling of LC with (tandem) mass spectrometry ((MS/)MS). This combination gives an efficient separation and excellent identification of individual compounds. Generally, chromatographic

separation of phytoestrogens is carried out with a reversed-phase (RP) C-18 column with a mobile phase of methanol and/or acetonitrile and water, all of them containing a small amount of an acid as modifier. Those modifiers (*e.g.* formic acid or acetic acid or trifluoroacetic acid), enhance the dissociation of phytoestrogens and their metabolites in a solvent system, and thus other parameters such as the separation, resolution and peak shape. Concomitantly, the addition of volatile additives also favors the ionization efficiency in the interface of the MS detector [96, 99].

In the late 1980s, primarily a thermospray (TSP) interface was used in LC-MS analyses and was also applied for the analysis of phytoestrogens by Setchell *et al* [100]. Due to some practical limitations of robustness and stability of this type of source, atmospheric pressure ionization methods like electrospray and atmospheric pressure chemical ionization are nowadays the state-of-the-art. Phytoestrogens are relatively polar to apolar compounds, and the aglycones have a low molecular weight (< 1000 amu) and can be ionized through APCI as well as ESI [101].

ESI has been dominating the field of MS detection for the determination of phytoestrogens. Little fragmentation of the analyte occurs with this technique, as ions are already formed in the solution that enters the source. The application of an electrical field then generates nebulization of the eluens and after drying by heated nitrogen gas or infrared lights, the formed ions enter a capillary and are attracted by the mass analyzer. Different from ESI, in APCI the ionization takes place chemically in the gas phase. A corona discharge needle, which generates a flow of electrons initiates this process. Charge transfer occurs over the nitrogen to the solvent molecules, to the compounds of interest. APCI can be applied to molecules of diverse polarity, on the condition that they are volatile and thermally stable. Generally protonated $[M + H]^+$ or deprotonated $[M - H]^-$ ions are formed , for positive or negative mode MS, respectively. Formation of simple adduct ions $[M + Na]^+$ or $[M + NH_4]^+$ and $[M + H - H_2O]^-$ occasionally occurs too [99].

Next, the mass analyzer, mostly a quadrupole MS analyzer, sorts the formed ions on the basis of their mass to charge ratio (*m/z*). The use of multiple MS instruments, like the very popular triple quadrupole (Figure 1.7), leads to very detailed structural information (MS/MS spectrum) and highly selective detection of phytoestrogens, both for identification and quantification. By performing mass spectrometry in multiple reaction ion monitoring or MRM, a highly sensitive and selective method can be developed for the analysis of target compounds. The advanced triple quadrupole detector allows the selective introduction of ions with a specific mass-to-charge value, which are induced to fragment in the second quadrupole (collision induced dissociation or CID) and the daughter fragment ions are then analyzed in the third analyzer. This approach is most frequently used in the analysis of phytoestrogens.

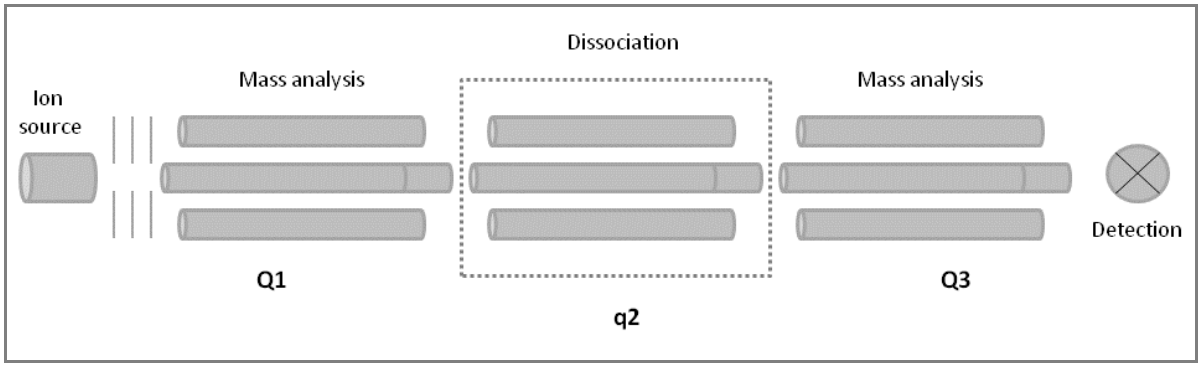



**Figure 1.7:** **A triple quadrupole detector for MS/MS analysis.**

Although the use of LC-MS(/MS) in the analysis of phytoestrogens has many advantages, one major limitation has to be considered, that is the effect of co-eluting residual matrix components on the ionization in API interfaces, the so-called **matrix effect** [102]. Typically, this effect results either in signal suppression or enhancement and concomitantly affects method performance parameters like limit of detection and quantification, accuracy, precision and linearity. Ion suppression or enhancement depends mainly on the sample matrix, exogenous materials (sample tubes, anti-coagulants), chromatography, mobile phase additives and the ionization mode (ESI > APCI) [103].

Unfortunately, the underlying mechanism of matrix effect is not yet fully resolved, and even the source design of the mass spectrometer can play a role [104]. This issue has already been highlighted and discussed by many authors and various suggestions have been presented to minimize it in LC-MS analysis: (i) a more selective and extensive sample preparation, which of course is time-consuming and increases the risk of losses during the several consecutive steps [105]; (ii) improvement of the HPLC separation efficiency [106]; (iii) dilution of extracts or decreasing the injected amount of sample [107]; (iv) decreasing the flow rate [108]. If aforementioned approaches do not eliminate the matrix effects, compensation by an appropriate calibration technique is mandatory [109]. This can be done by the following options: (i) internal standard calibration with structural analogues or labeled internal standards, although the latter also have been reported to suppress analyte ionization and are not always commercially available or are quite expensive; (ii) matrix matched standards, which is often not possible due to the lack of appropriate matrices and (ii) standard addition, which is though labour-intensive and time-consuming, but the ultimate solution if previous alternatives are not possible. Less common analytical solutions have also been suggested, *e.g.* echopeak injection of standards (injection of standard in close proximity of the analytes) and the use of a nano-splitting device or continuous post-column infusion.

For the evaluation of matrix effects during LC-MS(/MS) method development and validation Matuszewski *et al.* [103, 110] have published a valuable strategic approach. It involves the determination of peak areas for the analyte in two different sets of samples, one consisting of neat standards, resulting in reference peak area(s) and one prepared in blank matrix extract which is spiked after extraction. By comparing those sets of quantitative data, the matrix effect can be calculated and expressed.

## *2.4 Immunochemical techniques*

The basis of all immunochemical techniques is the reversible interaction of an antibody with its corresponding antigen, which is a highly specific and mostly of high affinity. In research, antibodies can serve as key molecules to study almost any interesting molecule, which leads to a large possible variation in assay specificity. Antibodies can be implied in screening analysis for both single and simultaneous measurement of multiple analytes, for the elucidation of the subcellular localization of an antigen, the isolation of the antigen from a mixture of molecules or to study other molecules that interact with the antigen. Immunochemistry opens up possibilities for the implementation of simple, rapid, robust, yet sensitive and easy applicable routine analyses in clinical laboratories [111].

- **Antibody structure**

Antibodies or immunoglobulins (Ig), function as a part of the immune system and are produced by B-lymphocytes in response to the presence of foreign molecules. They consist of a large family of host glycoproteins visualized as forming a typical Y-shape and perform two key functional roles: (i) they bind an antigen with their antigen binding sites, known as Fab fragments (or antigen binding fragments) and (ii) can serve as linkers between effectors of the immune system and specific antigens through their Fc fragment (or complement-binding fragment). Each antibody contains two identical copies of a light chain polypeptide (Mw of 25 kDa) and two identical copies of a polypeptide known as the heavy chain (Mw of 50 kDa). Each light chain consists of one variable VL and one constant domain CL and each heavy chain of one variable VH and three constant domains, CH1, CH2 and CH3. The four chains are held together with disulfide bridges and non-covalent bonds like salt bridges and hydrogen bonds. A hinge segment between the Fab and Fc fragments allows lateral and rotational movement of the two antigen binding domains, and consequently a flexible interaction of the antigen binding domains with various antigen conformations [111, 112].

On the basis of the number of Y-like units and type of heavy chain (γ, μ, α, ε, δ) five classes of immunoglobulins can be distinguished: IgG, IgM, IgA, IgE and IgD. The IgG class, is divided into four subclasses (often referred to as isotypes; $IgG_1$, $IgG_{2a}$, $IgG_{2b}$ and IgG3) and forms the most abundant one in serum. IgD, IgE and IgG are each made up of a single structural unit. IgA, on the other hand may contain either one or two units and IgM antibodies consist of five disulfide-linked structural units. All known antibodies contain either kappa (κ) or lambda (λ) light chains and any antibody will have only one type of light chain and one type of heavy chain [111, 112] (Figure 1.8).

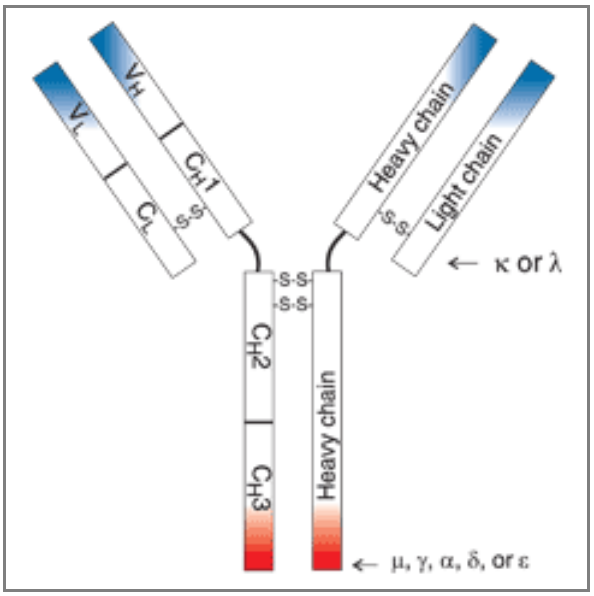



**Figure 1.8:** Structure of an immunoglobulin [113].

Antibodies interact with epitopes or immunodeterminant regions of antigens. The molecules that evoke antibody production must harbor a degree of intrinsic structural complexity which is named as immunogenicity. Macromolecules such as proteins or carbohydrates or combinations of these, which have molecular weights greater than 1000 Da or more, are natural immunogens that can evoke an immune response quite easily. Smaller structures like steroids, substituted aromatic groups and peptides, also called

haptens, have to be covalently linked to larger carriers before they can induce immune responses [114].

The reversible antigen-antibody (Ag-Ab) binding can be characterized using the parameters affinity and avidity. The strength of the binding is expressed by affinity and in terms of concentration Ag-Ab complex at equilibrium (typically from micro- to picomolar). The overall stability of the Ag-Ab complex which is determined by the antibody's affinity for the epitope, the number of binding sites per antibody and the geometric arrangement, is a measure for the avidity. Cross-reactivity of antibodies may influence the degree of specificity, but on the other hand it makes the immunochemical method applicable in situations where a class of structurally related molecules is targeted [111].

- **Polyclonal and monoclonal antibodies**

A polyclonal antiserum can be generated by the immunization of an animal (usually a rabbit, sheep or goat) and contains several immunoglobulins of different types, which can bind at various epitopes of the structurally complex immunogen. Apart from the capacity to form large insoluble immune complexes with the antigen and the ability to identify the antigen by the mixture of specificities, the use of polyclonal antibodies (pAbs) in immunoassays has certain limitations. These are mainly the non-uniform characteristics from batch to batch and between animals, and the heterogeneity at many levels: specificity, classes and isotypes of the antibody, affinity and reactivity [112].

The limitations of polyclonal antibodies have led to the first attempt to produce monospecific monoclonal antibodies (mAbs), by Köhler and Milstein in 1975. They have identical physical, biochemical, and immunological properties and each monoclonal product is specific to a single antigen determinant of the immunogen (monospecific). Monoclonal antibodies are derived from single-antibody-producing cells immortalized by fusion to myeloma cells (a type of B-cell tumor) to form hybrid cells or hybridomas. After the selection

in a particular culture medium and screening, an *in vitro* cell line clone which produces a specific mAb is obtained. Monoclonal antibodies are powerful immunochemical tools with several advantages: (i) they constitute a well-defined reagent derived from one isolated clone, (ii) may be prepared from non-purified antigens, (iii) unlimited quantities of the same homogeneous reagent can be produced and (iv) their affinity and specificity are well-defined [112].

Most commonly BALB/c mice are used for the immunization, as many of the first examples of myelomas (referred to as MOPC or mineral oil plasmacytoma) were isolated from these mice by intraperitoneal injection of mineral oil. Nowadays, derivatives of those cells, such as Sp2/0 and NS0 have become the most used partners for fusions. The antibody-secreting cells are prepared from immunized animals which are injected with an antigen preparation and developed a good humoral response. Polyethylene glycol (PEG) is used as a fusing agent for the production of the hybridomas and fuses the plasma membranes of adjacent cells to form a single cell with two or more nuclei. Mitosis of this heterokaryon and consequent dissolving of the nuclear membranes, is then followed by the segregation of the individual chromosomes into daughter cells during further rounds of division. Hybridoma fusion process is not very efficient and a large number of unfused cells remain in the culture. Therefore, unfused myeloma cells are eliminated by drug selection. The addition of aminopterin (A) to the medium of the hybrids, blocks the *de novo* biosynthesis of purines and pyrimidines, essential for DNA synthesis. Cells will then be forced to use the salvage pathway utilizing hypoxanthine (H) and thymidine (T) and require a functional thymidine kinase (TK) and HPRT; therefore, this allows only fusions of myelomas with a non-functional hypoxanthine phosphoribosyltransferase (HPRT) and cells with a functional HPRT to grow. The fusion products are diluted in this selective HAT medium and plated out in multiwell tissue culture dishes. After testing of the hybridoma supernatant for the requested mAb production in dedicated enzyme-linked immunosorbent assays (ELISA), cells from positive wells are subjected to several rounds of limiting dilution in order to attain single-cell clones, which can then be screened and further expanded. The final and stable hybridomas are frozen for storage and in this way an unlimited supply of mAbs can be maintained. This

whole production can take less than 2 months but can also take more than a year, as every stage has its inherent problems which have to be dealt with [112, 114].

- **Immunoassays**

The most popular and powerful of all immunochemical techniques are the immunoassays, which can be divided into three classes, (i) the antibody capture or indirect competitive immunoassays, (ii) the antigen capture or direct competitive immunoassays and (iii) the two-antibody or sandwich immunoassays. In a heterogeneous system, at least one separation step is involved to distinguish reacted from unreacted material, and those immunoassays that do not require separation are referred to as homogenous immunoassays [112].

In the antibody capture assay (Figure 1.9), the antigen is attached to a solid support and the sample is added together with a constant and limited amount of labeled antibodies specific for the antigen. After washing, the quantitation is done by measuring retained antibody and the signal is inversely related to the concentration of antigen/standard in the sample. The detection can be performed in a one-step format with labeled antibody, but the two-step format, which makes use of a secondary labeled antibody specific for the bound antibody, provides several fold improved assay sensitivity.

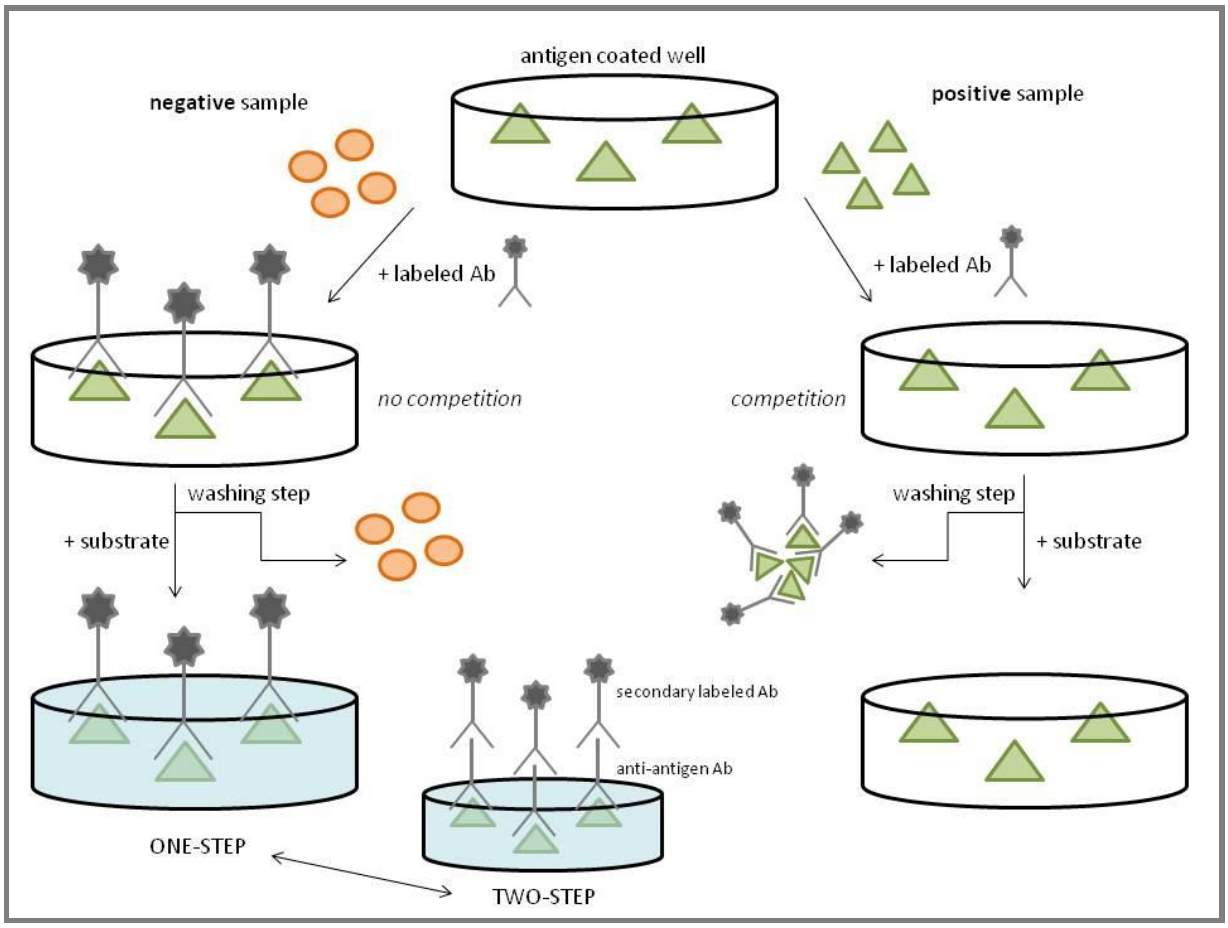



**Figure 1.9:** **Set-up of an antibody capture or indirect competitive immunoassay (competition < positive sample; no competition < negative sample in a one-step versus two-step format with color development).**

In the second and third type of assay a specific primary (or secondary IgG-specific antibody) is physically adsorbed or covalently attached to the solid support and unattached antibody is washed away.

Then, in the direct competitive assay or antigen capture assay, in the presence of standard or sample antigen to be assayed, the quantitation is performed by measuring retained purified labeled antigen that was added (Figure 1.10).

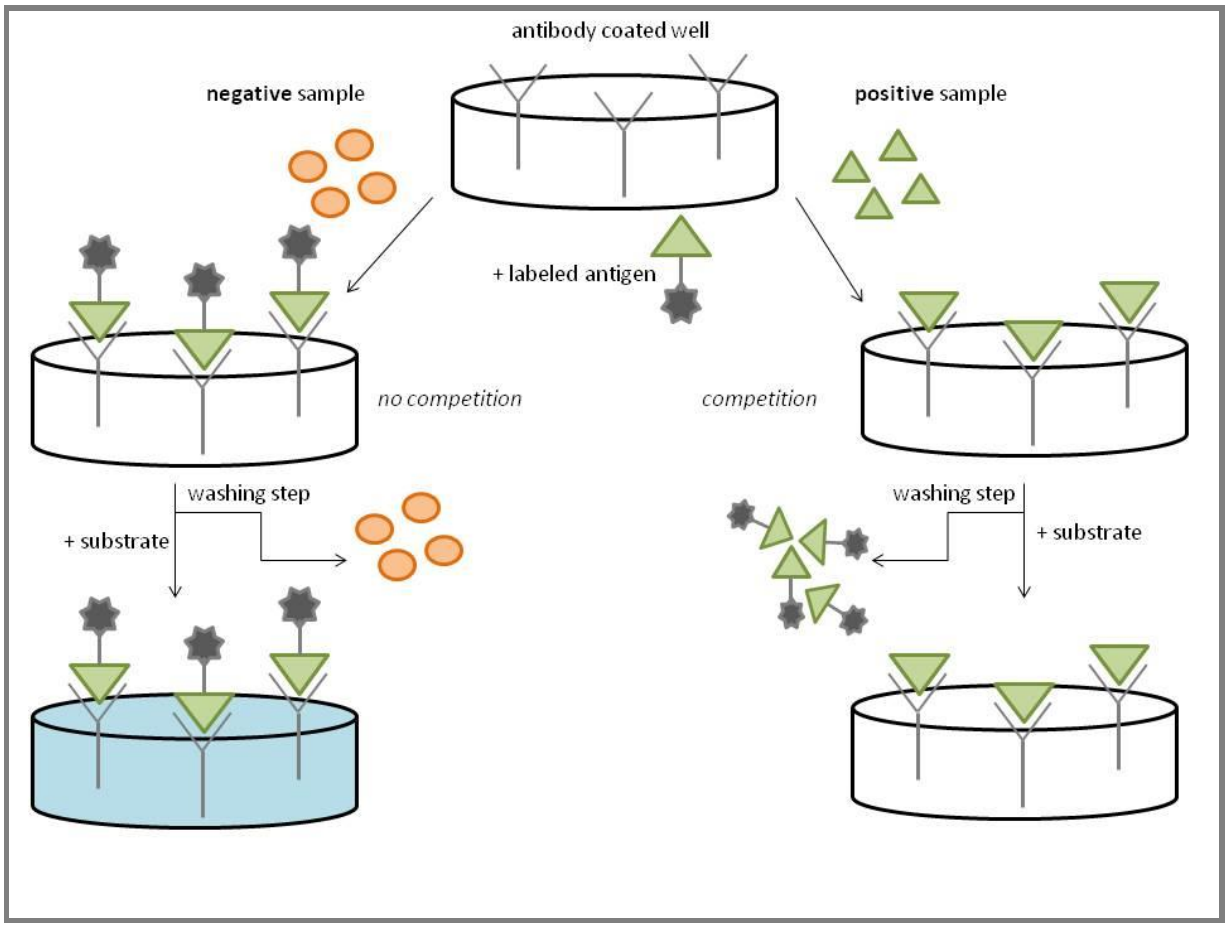



**Figure 1.10:** **Set-up of an antigen capture assay or direct competitive assay (competition < positive sample with color development; no competition < negative sample).**

In the popular sandwich immunoassay, which is primarily used to determine antigen concentrations, the binding of two antibodies occurs, either sequentially or simultaneously. For this purpose, multiple non-overlapping epitopes on the antigen are a requirement. A primary antibody recognizing an epitope of the molecule to be detected or an anti-Ig antibody can be immobilized to a solid phase. After incubation of the sample with the immobilized antibody and reaction with the first epitope, the solid phase is washed to remove unreacted components. The color reaction can be evoked, either directly by an enzyme-conjugated secondary antibody (Figure 1.11), or indirectly, by a third conjugated antibody against the Fc-portion of the second antibody [111]. The signal is proportional to the level of labeled detector antibody that is bound to the antibody-antigen complex.

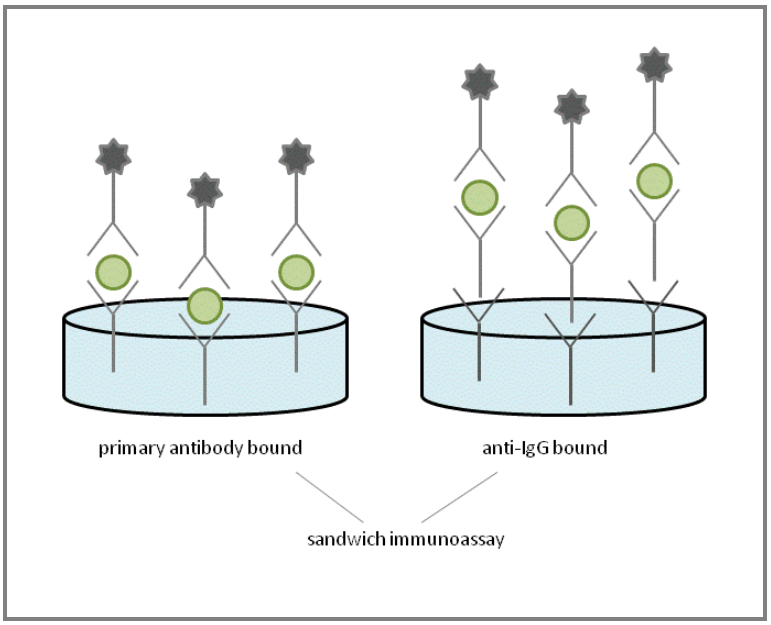



**Figure 1.11:** Two types of sandwich assay format.

Several possible formats of immunoassay can be performed with four variations in the assay, *i.e.* an assay in antibody excess, in antigen excess, as an antibody competition, or as an antigen competition [112].

In immunoassays, the used proteins, which are antigens or antibodies, can be labeled with radioactive compounds, enzymes, biotin or fluorochromes. Radioimmunoassays (RIA) have been used in the beginning years of immunoassay application, but are now often replaced by the quicker and nonhazardous alternative using enzyme-conjugated labels, the enzyme immunoassay (EIA), such as the enzyme-linked immunosorbent assay (ELISA). The enzymes most widely used are horseradish peroxidase (HRP) and alkaline phosphatase (AP). A variety of aromatic phenols or amines, such as ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), TMB (3,3',5,5'-tetramethylbenzidine) or OPD (*O*-phenylethyldiamine) can be used as chromogenic substrates for HRP, and for AP the typical substrate used is *p*-nitrophenylphophate (*p*NPP). The labeling enzymes convert a colorless substrate to a colored end-product, which is being visualized in a spectrophotometer at a specific absorbance maximum and quantitated [111, 112, 115].

- **Immunostaining (immunocytochemistry)**

Immunostaining, a technique first introduced by Coons *et al.* in 1941 [116], applies a labeled antibody to detect a specific protein or antigen in a sample (cells or tissues). Moreover, it will demonstrate both the presence and the subcellular localization of an antigen. Often cell staining is combined with conventional histological stains for the comparison of the localization of the antigen with other markers. Immunostaining techniques are conducted in four steps: (i) cell or tissue preparation, (ii) fixation, (iii) antibody binding and (iv) detection. The outcome of the immunostaining procedure or how easily the antigen is detected, depends on each of those steps, and consequently needs fine optimization. The local antigen concentration influences the signal strength and diffuse antigens are more difficult to detect than areas with concentrated antibody binding sites. The fixation is important for the preservation of cell morphology and tissue. Ideally, the fixation does not mask or alter the epitopes too much, and retains the authentic cellular and subcellular architecture. Hence, the type of antibody used to recognize the epitope(s) can also play an important role. For the antibody detection, mostly fluorescent markers are applied. They offer the advantage of a rapid visualization and improve assay sensitivity. Moreover, the use of new types of fluorophores diminishes the problem of photobleaching over time [111, 112]. Their advantage of speed and simplicity has made that the use of fluorescent labeling remains more popular than enzymatic labels. The availability of labeled antibodies with different emission spectra also offers the possibility to detect two or more antigens in the same experiment [117].

- **Immunoprecipitation**

Another useful application of specific antibodies is immunoprecipitation (IP), which is used to isolate and concentrate a certain antigen (usually a protein) out of a sample. A number of important characteristics of the antigen, like presence and quantity, relative molecular weight, rate of synthesis or degradation, presence of certain post-translational modifications, and interactions with other ligands, can be determined, especially when IP is

coupled to SDS-polyacrylamide gel electrophoresis. A protein mixture of the conditioned cells or tissue is made by lysis with detergent, to release the targeted antigens. Then, in the direct way, antibodies which are immobilized on (agarose) beads are added to the mixture and the antigens are captured onto the beads, via the antibodies. On the other hand, in the indirect procedure, antibodies are pre-incubated with the protein mixture to form Ab-Ag complexes. Then afterwards, protein A/G coated beads are added, to capture the antibodies with corresponding bound specific antigens. Finally, the immune complexes are purified from the bulk mixture and can then analyzed by gel electrophoresis, possibly followed by sequencing using MALDI mass spectrometry. Or, the gel can be transferred in Western Blot with other antibodies interacting with the antigen. IP combined with those other techniques can be used to gather a large amount of information about the antigen [112, 118].

# CHAPTER 2

## Objectives

*AIMS AND OUTLINE OF THE THESIS*

At each level of the research on phytoestrogens, from the original plant to the dietary supplement and finally the biological sample, an accurate, precise and reliable method for determination is mostly wanted. It is a necessary tool in the correlation of epidemiological and clinical intervention data with health outcome. Since the growing interest in the phytoestrogens and their application in the clinical environment, mainly as dietary supplement by peri- and postmenopausal women for the relief of vasomotor symptoms, the range of methods has broadly expanded.

The **determination of phytoestrogens** in biological samples, either qualitatively or quantitatively, requires an optimized procedure, mostly unique for the sample, the target compounds and the used analytical method. Nevertheless, the growing laboratory costs for both staff and materials, necessitate the development of simple, but efficient protocols, which is a very challenging task. This starts with a dedicated clean-up procedure of the sample, which depends on the origin of the sample, the target compound(s) and the used analytical method. Initially, phytoestrogens were analyzed using imprecise and insensitive techniques, such as thin layer and paper chromatography. Then, in the mid-1980s, the principal method for phytoestrogen analysis was gas chromatography coupled with mass spectrometry (GC-MS), a technique which is nowadays still being used, although it is very labor intensive. More commonly applied, mainly because it requires less sample preparation and is a relatively rapid way to separate and quantify phytoestrogens, is high performance liquid chromatography (HPLC) with ultraviolet detection (UV). When more sensitivity is required, to measure concentrations of less than parts per million, the ultimate solution is HPLC coupled with (tandem) MS. The latter requires a less destructive sample preparation than for GC-MS, where conjugating groups must be removed prior to analysis. Nevertheless, the measurement stays dependent on the availability of internal standards for each analyte and the ability to produce molecular ions. Ultimately, there is a general and great demand for more rapid forms of analysis, and the non-chromatographic immunoassay techniques may offer a reasonable solution for this problem.

This PhD started as part of an integrated project on phytoestrogens present in the diet or in nutritional supplements (acron: FYTOES). The contracted research, which was supported by the Federal Public Services, department of Health, Safety of the Food Chain and Environment, aimed to study the biological availability, the physiological and toxicological impact of phytoestrogens with respect to human health. Moreover, the aim of the research was to get an estimation of the possible toxic effects, acute and chronic biological effects linked with the intake of phytoestrogens.

At the beginning of this research project profound literature review (see Appendix; table A.1) demonstrated a clear demand for accurate and sensitive quantification methods for phytoestrogens in biological samples. Although both LC-MS and the more sensitive LC-tandem MS methods were successfully applied for the simultaneous determination of isoflavones and lignans in urine and serum, none of them also included the hop-derived prenylflavonoids. Also, the majority of the reported methods for the quantitation of prenylflavonoids in biological samples focused on HPLC-UV instead of the more sensitive LC-MS. Therefore, the first objective and challenge was to **develop and optimize a selective and sensitive analytical method based on high performance liquid chromatography–mass spectrometry (LC-MS) for the determination of thirteen phytoestrogens including their gut metabolites.** Furthermore, this method had to be validated for its use in the quantification of the phytoestrogens in urine and serum. Next, the aim was to apply the method for high-throughput quantitative analysis of biological samples generated in an intervention dose-related study using phytoestrogen-containing products in test subjects. Moreover, this study included a co-supplementation phase (administration of isoaflavones, lignans and prenylflavonoids) and the developed method aimed to simultaneously measure all the phytoestrogens in one analytical run.

The second objective was to **produce monoclonal antibodies against a selected group of phytoestrogens** (genistein, daidzein, equol, coumestrol, enterodiol, enterolactone, xanthohumol, isoxanthohumol and 8-prenylnaringenin) and to **apply them in a newly developed enzyme linked immunosorbent assay (ELISA)**. Furthermore, this ELISA test had

to be validated for its use in the analysis of urine and serum samples. Immunoassays applying polyclonal antibodies had already been successfully generated for both isoflavones, enterolignans and coumestrol (see Appendix: table A.2). To the best of our knowledge only monoclonal antibodies against daidzein, genistein and equol were available at the start of this PhD.

A third objective of this thesis was to **explore the possibility of using the developed antibodies in other immunochemical techniques**, such as immunocytochemistry and immunoprecipitation. Those techniques were used for the determination of subcellular and molecular interactions of the hop-derived prenylchalcone xanthohumol.

# CHAPTER 3

## Selective and sensitive detection of phytoestrogens with HPLC-MS

### METHOD DEVELOPMENT, OPTIMIZATION AND VALIDATION

This chapter is partially based on:

*Development of a high-throughput LC/APCI-MS method for the determination of thirteen phytoestrogens including gut microbial metabolites in human urine and serum.*

Wyns, C., Bolca, S., De Keukeleire, D. and Heyerick, A.

Journal of Chromatography B, 2010. 878(13-14): p. 949-956.

# 1. INTRODUCTION AND OBJECTIVES

The investigation into the potential usefulness of phytoestrogens in the treatment of menopausal symptoms requires large-scale clinical trials that involve rapid, validated assays for the characterization and quantification of the phytoestrogenic precursors and their metabolites in biological matrices, as large inter-individual differences in metabolism and bioavailability have been reported. Since the beginning of research on phytoestrogens and their metabolites in a great diversity of matrices, from plants to human biological fluids and tissues, a wide range of analytical techniques for their determination and quantification have been reported. As reviewed by several authors [96, 99] the techniques used can be classified into chromatographic and non-chromatographic, with high-performance liquid chromatography (HPLC) and gas chromatography (GC) as main tools in the former group and immunoassay techniques in the latter group. Generally, chromatographic separation is coupled to detection techniques like ultraviolet (UV) and mass spectrometry (MS). Most reported chromatographic methods cover detection and quantification of a limited number of phytoestrogens and/or their intestinal metabolites in biological fluids [119, 120]. Moreover, the greater part of the techniques require expensive equipment like a tandem mass spectrometer and analyses are often preceded by an intensive multi-step clean-up and complex sample preparation procedures such as derivatization to increase compound volatility for GC [121-123].

This chapter describes how the development, optimization and validation of a new sensitive high-performance liquid chromatography-mass spectrometry method (HPLC-MS) for the quantitative and simultaneous determination of thirteen phytoestrogens including their most important gut microbial metabolites (genistein, daidzein, equol, dihydrodaidzein, *O*-desmethylangolensin, coumestrol, secoisolariciresinol, matairesinol, enterodiol, enterolactone, isoxanthohumol, xanthohumol and 8-prenylnaringenin) in human urine and serum was performed.

In the **first part** the development of a new and selective chromatographic method for the separation of 13 phytoestrogens is explained. A simple sample preparation procedure is used, consisting of enzymatic deconjugation using *Helix pomatia* β-glucuronidase/sulfatase followed by organic liquid-liquid extraction (LLE) or solid-phase extraction (SPE) for urine or serum, respectively.

In a **second part** the sensitivity of the mass detection of the phytoestrogens and their metabolites was optimized with an Agilent Multimode Source (MSD) SL (Superior Line) 1200 single quadrupole mass spectrometer (Figure 3.1). This ion source is a MS-source, which can simultaneously generate APCI-ions and ESI-ions (mixed mode) and can operate both in the positive and negative mode. The SL-type also offers the possibility to set 4 MSD-signals, which can solve problems of poor LC resolution for some compounds. For quantitative analyses preferably selective ion monitoring or SIM-mode is used. The latter influences detection sensitivity, but also the ionisation method and other settings of the ionisation source are important parameters. Using Flow Injection Analysis (FIA) (which introduces the sample directly in the mass detector), the optimal settings of the MSD were obtained.

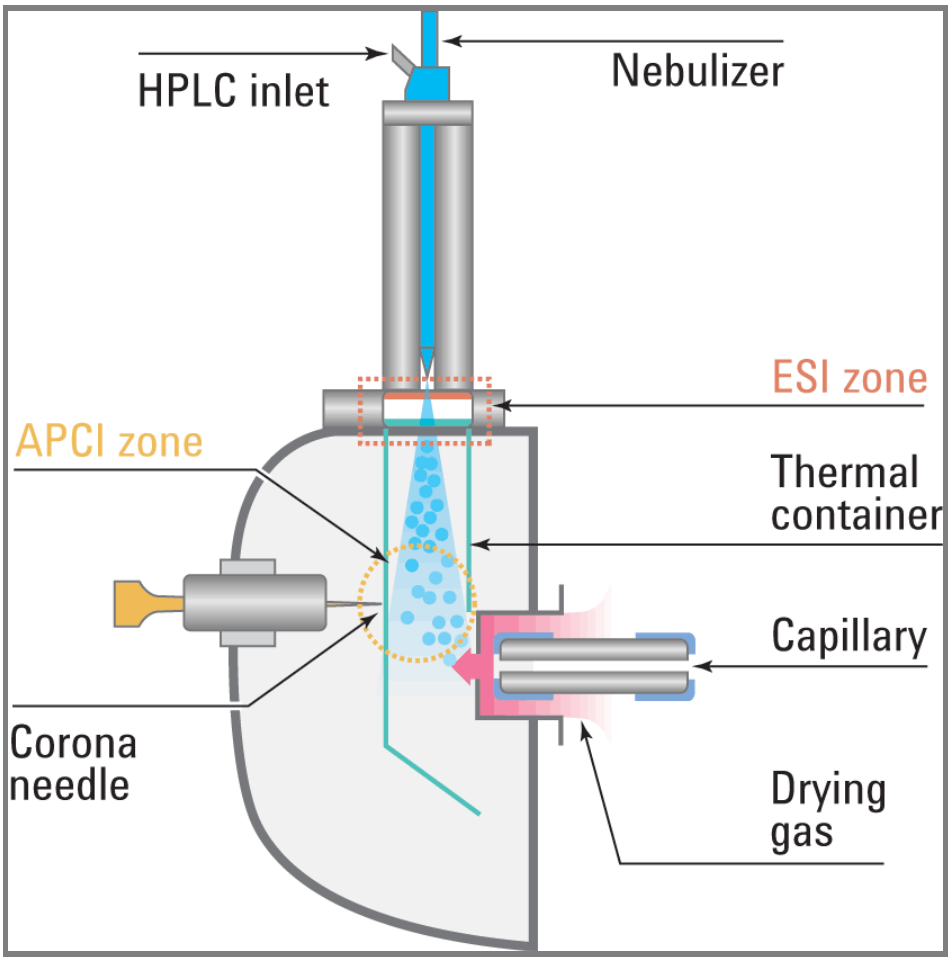



**Figure 3.1:** Agilent multimode ion source [124].

The third part decribes the full validation of the bioanalytical method following the guidance for Industry – Bioanalytical Method Validation recommended by the Food and Drug Administration of the United States. Validation is conducted in order to confirm that the developed method is suitable for the quantification of a concentration of a certain analyte in a certain biological matrix. It has to be proved to allow an accurate and precise quantification of the targeted phytoestrogens and their metabolites covering the lower parts-per-billion range for the measurement of relevant urine and serum levels following ingestion of phytoestrogen-rich dietary supplements. This method was developed for the analysis of samples collected in a clinical trial conducted by the same laboratory in collaboration with the Ghent University Hospital, but can also serve as a guiding principle for other research groups in the domain of phytoestrogens.

# 2. EXPERIMENTAL

## *2.1 Chemicals and reagents*

Isoxanthohumol (IX), xanthohumol (X(N)) and 8-prenylnaringenin (8-PN) were available in our laboratory [52]. Daidzein (DAID) en genistein (GEN) were purchased from Acros Organics (Morris Plains, NJ, USA), equol (EQ) from Extrasynthèse (Genay, France) and dihydrodaidzein (DHD) and *O*-desmethylangolensin (*O*-DMA) from Plantech UK (Reading, UK). Secoisolariciresinol (SECO), matairesinol (MAT), enterodiol (END), enterolactone (ENL), coumestrol (COUM), the internal standard 4-hydroxybenzophenone (4-HBPH) and β-glucuronidase/sulfatase from *Helix pomatia* (Type H1) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All reference standards had a minimum HPLC purity of 95%, except for matairesinol (85%). Sodium acetate was obtained from UCB (Leuven, Belgium). Methanol, diethyl ether and *n*-hexane used during sample preparation were from ChemLab (Zedelgem, Belgium). Formic acid was purchased from Acros Organics. Water, methanol and acetonitrile used for chromatography were of LC-MS grade (Biosolve, Valkenswaard, The Netherlands).

## *2.2 Standards and samples*

Primary stock solutions of standards and internal standard were prepared in methanol at a concentration of 1.0 mg/mL and 0.2 mg/mL, respectively. From these, a working solution containing each of the thirteen standards was made at a concentration of 10 µg/mL for IX, XN, 8-PN and COUM, and 100 g/mL for DAID, GEN, DHD, *O*-DMA, EQ, SECO, MAT, END and ENL. This mixture and further dilutions were used for spiking of urine samples, whereas a 2:5 dilution was used for serum samples. All the above mentioned solutions were kept at -20 °C.

For method development and validation, pooled human blank urine was collected from volunteers in the laboratory and pooled human blank serum was purchased from Innovative Research (Novi, MI, USA). Blank urine and serum from different individuals were obtained from the dietary intervention trial (FYTOES) for which the method was developed. All samples were stored at -20 °C until analysis. Prior to extraction, urine samples were centrifuged at 3000 rpm for 10 minutes to remove solids and the serum was defatted using *n*-hexane (1:1; v/v).

### *2.3 Preparation of calibration standards and quality control samples*

Calibration standards (calibrators), except blanks, were prepared by spiking with the standard mixture to give concentrations for IX, XN, 8-PN and COUM ranging from 0.1 to 400.0 ng/mL in urine, and 0.04 to 100.0 ng/mL in serum; for DAID, GEN, DHD, *O*-DMA, EQ, SECO, MAT, END and ENL, final concentrations in urine ranged from 1.0 to 4000 ng/mL and in serum from 0.4 to 1000 ng/mL. The added volume was less than 5% of the total volume of the samples in order to maintain the integrity of matrix. Quality control (QC) samples were freshly and independently prepared at three levels (low, medium and high). Moreover, DAID, GEN, DHD, *O*-DMA, EQ, SECO, MAT, END and ENL were spiked in urine at 800 ng/mL for low QC level, at 2000 ng/mL for medium QC level, and at 3000 ng/mL for high QC level, and IX, XN, 8-PN and COUM were spiked at 80 ng/mL for low QC level, at 200 ng/mL for medium QC level, and at 300 ng/mL for high QC level. In serum, a 1:5 dilution of the three QC level spike solutions was used. All these prepared solutions were aliquoted and stored at -20 °C until required.

## *2.4 Preparation of samples*

- **Urine**

Urine sample preparation was based on a simple protocol previously published by Bolca *et al.* [125]. Prior to extraction, thawed urine samples (2.0 mL) were diluted with an equal volume of sodium acetate buffer (pH 5.0; 0.1 M), spiked with 100 µL internal standard (20 µg/mL) and 30 µL of deconjugation mixture (*Helix pomatia* 33 mg/mL; β-glucuronidase activity: 300 U/mg and sulfatase activity: 15.3 U/mg) and then incubated for one hour at 37 °C. After incubation, a liquid-liquid extraction (LLE) was carried out (twice) using 5 mL diethyl ether and subsequent vortex mixing (30 s). The collected organic solvent layers (5 mL (2 x 2.5 mL) in total) were dried under a gentle nitrogen stream, dissolved in 100 µL injection solvent and transferred into a vial for LC-MS analysis. The injection volume was set at 25 µL.

- **Serum**

For the extraction of the target compounds from the serum samples, a standard protocol that makes use of solid-phase extraction (SPE) was evaluated and optimized. The final method is summarized as follows: 800 µL of each sample was transferred to an eppendorf tube, diluted with an equal volume of sodium acetate buffer (pH 5.0; 0.1 M), and spiked with 50 µL internal standard and 800 µL deconjugation mixture and then incubated at 37 °C overnight. Bond Elut C18 SPE cartridges were preconditioned with 3 mL methanol and 3 mL sodium acetate buffer prior to use. The sample was applied and after washing with 3 mL aqueous methanol (5%) and 5 minutes drying of the sorbent under vacuum, the target compounds were eluted with 3 mL methanol. This was followed by drying of the eluents under a gentle stream of nitrogen and dissolving in the injection solvent. An injection volume of 25 µL or 50 µL was applied.

## 2.5 Instrumentation

Method development, optimization and validation were conducted on an Agilent Technologies (AT, Santa Clara, CA, USA) 1200 series LC, equipped with a binary gradient pump, autosampler, thermostatted column oven and photodiode array UV detector. Chromatographic separation of all compounds in one single run was performed with a Waters XBridge C18 reversed phase (3.5 µm) column (3.0 mm id x 150 mm column) connected to a 3.0 mm id x 20 mm C18 guard column (3.5 µm) and maintained at a temperature of 55 °C. The mobile phase was degassed by the integrated AT 1200 series vacuum degasser. The mobile phase consisted of $H_2O$ (eluent A) and MeOH/MeCN (80:20, w/w) (eluent B), both acidified with 0.025% (v/v) formic acid. The gradient and flow rate were programmed as follows: 0-3.5 min: 35% to 45% B (flow: 0.6 mL/min), 3.5-5.5 min: isocratic 45% B (flow: 0.6 mL/min), 5.5-13 min: 45% to 100% B (flow: 0.6 mL/min), 13-16 min: isocratic 100% B (flow: 0.8 mL/min), 16-16.10 min: 100% to 35% B (flow: 0.6 mL/min) and 3.9 min for re-equilibration of the column.

MS analysis was performed using an AT multimode ionization source coupled to a single quadrupole detector (MSD), SL version. A standard APPI/APCI calibration mix was used for daily tuning of the MSD and processing of the data was carried out using the LC/MSD Chemstation software (Rev. B.02.01).

## 2.6 Method development

The development of a selective and sensitive analytical method for phytoestrogens was completed in two phases. In the first phase a selective chromatographic method for the separation of 13 phytoestrogens and their metabolites was optimized. Three important aspects that determine the selectivity of a chromatographic separation were considered: the mobile phase, the stationary phase and the pH. The mobile phase was composed of eluent A and eluent B, which were respectively $H_2O$ and a mixture of MeOH and MeCN. The addition of different concentrations of formic acid or trifluoroacetic acid and their

influence on retention and separation was regarded. Traditionally for the separation of phytoestrogens hydrophobic stationary phases are used, like a C18 column. The second phase of the development was optimization of the sensitivity of mass detection using the Agilent MSD. For quantitative analyses Selective Ion Monitoring or SIM-mode is preferably used. The latter will influence the sensitivity of the detection, but also the ionization method and the settings of the ion source are important. For each compound separately and the internal standard, using Flow Injection Analysis (FIA), a technique which directly introduces the sample on the mass detector, the optimal settings of the MSD-parameters were determined.

### *2.7 Method validation*

The HPLC/APCI-MS method was fully validated following the guidance for industry of the US Food and Drug Administration (FDA) for the Validation of Bioanalytical Methods [126], with emphasis on the key elements described by Bansal *et al.* [127]. The following parameters were evaluated: selectivity, linearity, sensitivity, accuracy, inter- and intra-assay precision and stability. Additionally, recovery for the analytes of interest was calculated and an assessment of matrix effects was made in correspondence with the strategy applied by Matuszewski *et al.* [110]. SPSS for Windows version 17.0 was used to carry out all statistical analyses.

- **Selectivity**

The selectivity, *i.e.* the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample, was assessed by analyzing five different batches of blank urine and serum. Samples (blank and zero) were processed by the appropriate extraction procedure and the resulting MS chromatograms were evaluated for the presence of interferences at the retention times of the analytes and the internal standard.

- **Linearity and sensitivity**

Calibration curves were constructed over individually specified ranges using eight non-zero standard points in six replicates. In addition, a blank (non-spiked sample) and a zero sample (only spiked with IS) were run to exclude the presence of interferences. Peak area ratios between the analytes and the internal standard were plotted against the nominal concentrations of the calibration standards. Unweighted linear regression analysis generated calibration curves with the standard equation: $y = ax + b$, where $y$ is the peak area ratio, $x$ the concentration, $a$ the slope, and $b$ the intercept of the regression line. The adequacy of the assumed model and the linearity of the calibration curves was evaluated by observing correlation coefficients ($r^2$) and by visual inspection of the plots of the residuals (assumption of homoscedasticity). In addition, linearity was also assumed if the relative residuals did not exceed 15% and when at least 75% of the calibration standards met the stated acceptance criteria. Daily prepared calibration curves were constructed by injecting calibration standards at the beginning and at the end of each batch of samples. The limit of detection (LOD) and the limit of quantification (LOQ) of the method were defined as the lowest concentration with a signal-to-noise ratio of 3 and 10, respectively, in spiked samples, where the noise data were taken from the analysis of blank matrices.

- **Accuracy and precision**

The accuracy and the precision of the method were evaluated by analyzing QC samples at three levels (low, medium and high) ($n$=6) according to the procedure described above on four consecutive days. The accuracy (%RE) was calculated for each analyte in terms of correspondence of the measured mean value to the nominal concentration value. Analysis of variance (ANOVA) was used to calculate inter- and intra-assay precision for each QC level. To determine inter-assay variation (intermediate precision), the QC samples were analyzed at the three levels on four different days. The intra-assay variation (repeatability) was expressed for each QC level as the coefficients of variation (%CV) of measurements on

the same day. The accuracy and intra-assay precision values should be less than 15% and should not exceed 20% for the inter-assay variation.

- **Stability**

Two types of short-term stability experiments were performed at the low and high QC level. The autosampler stability was estimated on processed samples (*n*=3) at room temperature before and after an interval of approximately 15 h. The stability of the compounds was also examined during three freeze-thaw cycles at – 20 °C. Three replicates of spiked urine and serum samples were allowed to thaw at ambient temperature and were then refrozen for minimum 12 h. Aliquots of all samples were quantified at the end of the third freeze-thaw cycle. The concentration of each compound was compared to that of fresh samples and expressed in terms of degradation.

- **Recovery and matrix effect**

For the assessment of recoveries of the analytes and matrix effects caused by constituents in urine and serum, a strategy was applied which has previously been described by Matuszewski *et al.* [110]. The recovery was calculated as the ratio ((C/B)x100) between the responses of the QC samples in real blank matrix (low and high level, *n*=3) spiked after extraction (B) and before extraction (C) and is therefore not influenced by effects of the sample matrix. The absolute matrix effect (ME%) can be defined as the ratio of the responses between samples spiked after extraction (B) and pure standards in the mobile phase (A). When the value of this ratio ((B/A)x100) is < 100% there is signal suppression and signal enhancement is noted if the value is > 100%. Taking into account the heterogeneity of this effect between different sample batches, experiments were performed in six independent batches of blank urine and serum.

# 3. RESULTS AND DISCUSSION

## *3.1 Chromatographic and mass spectrometric method development*

First, the reversed chromatographic separation of the thirteen analytes of interest and the internal standard was optimized. The Waters XBridge C18 column was preferred over the Omnispher (Varian) and Xterra (Waters) column because of its high separation efficiency. A gradient elution method for the separation of 13 phytoestrogens and their metabolites and an internal standard was optimized by varying the amount of methanol and acetonitrile in eluent B from 100% methanol to 100% acetonitrile with steps of 5%. Starting from a basic gradient (30% B to 100% B in 30 minutes) the final gradient was developed (as described in section 2.5). The mobile phases were acidified to 0.025% formic acid. The type of acid does not influence chromatography directly, but can influence the response of the mass detector. Formic acid is preferred over trifluoroacetic acid, because the latter with its high surface tension can negatively influence the ionisation efficiency and sensitivity. At last the column temperature was varied from 30 °C to 60 °C and finally set at 55 °C, which resulted in the most efficient separation and a faster elution. Finally, our target compounds were eluted within 13 minutes with a resolution higher than 1.5, except for three components ($R_{equol,\ genistein}$= 1.13 and $R_{genistein,enterolactone}$= 1.06). After 20 minutes the column was washed and equilibrated for a next injection.

Although complete resolution of EQ, GEN and ENL on UV-detection was not accomplished with the optimized LC method, subsequent mass selective detection allowed full separation of these compounds. The interface and MS parameters were optimized individually for each component and are listed in Table 3.1. Atmospheric pressure chemical ionization (APCI) in the positive mode was selected, except for END and for ENL which gave the highest sensitivity with APCI in the negative mode. Pseudomolecular ions were predominant, with $[M+H]^+$ for the positive and $[M-H]^-$ for the negative mode. However, for SECO, the fragment ion $[M+H-2H_2O]^+$ was found to constitute the base peak (*i.e.*, 100%

relative intensity of the ion), in agreement with the observations by Bambagiotti-Alberti *et al*. [128] and Schmidt *et al*. [129]. Formation of this fragment ion is probably due to protonation of the γ-hydroxyl groups of SECO and subsequent loss of water from the unstable oxonium ions. In Figure 3.2, a typical mass spectrum of 8-prenylnaringenin is depicted (*m/z*= 340). Finally, using single-ion monitoring (SIM) and operating the MS in the chosen ionization method, the fragmentor voltage, a component-dependent parameter in MS sensitivity and possible initiator of collision-induced dissociation (CID), was varied from 50 V to 400 V. A typical FIA-analysis on fragmentor voltage for 8-prenylnaringenin in SIM-mode (pseudomolecular ion *m/z*= 341) and ionization through APCI+ is shown in Figure 3.3.

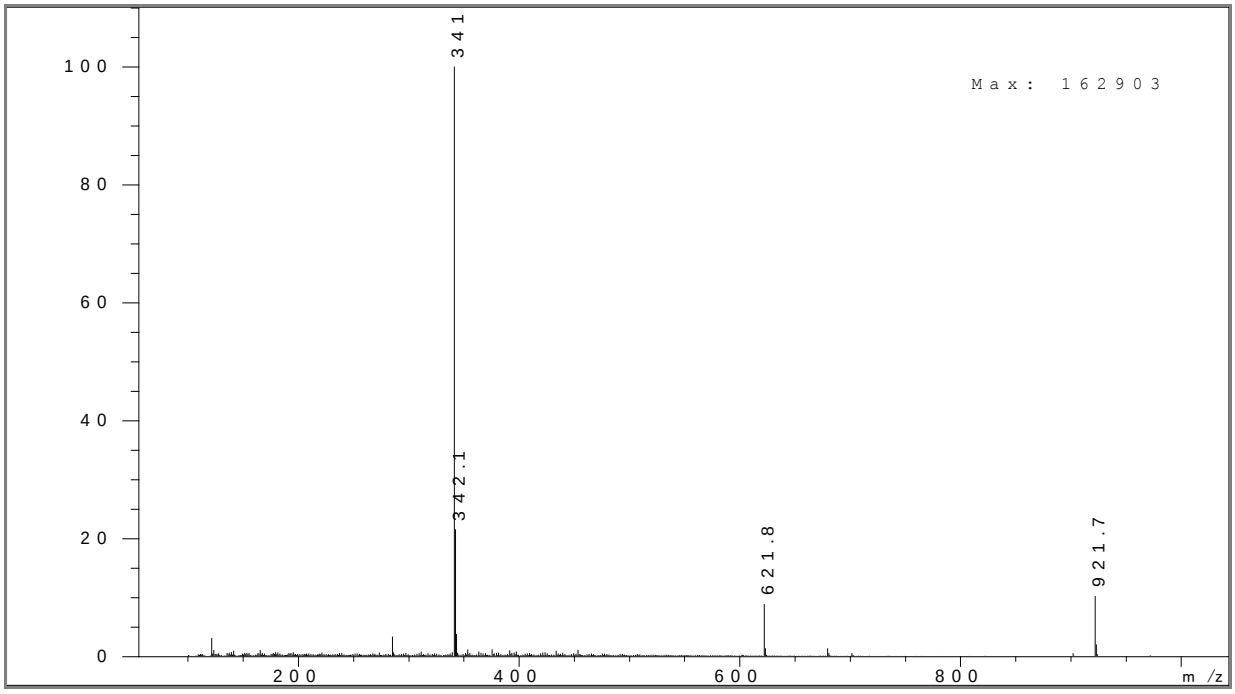



**Figure 3.2:** **Scan of 8-PN in APCI positive mode (*m/z* 100-1000; fragmentor voltage 150 V; standard solution 8-PN (1 mg/mL; 0,1 µL injection).**

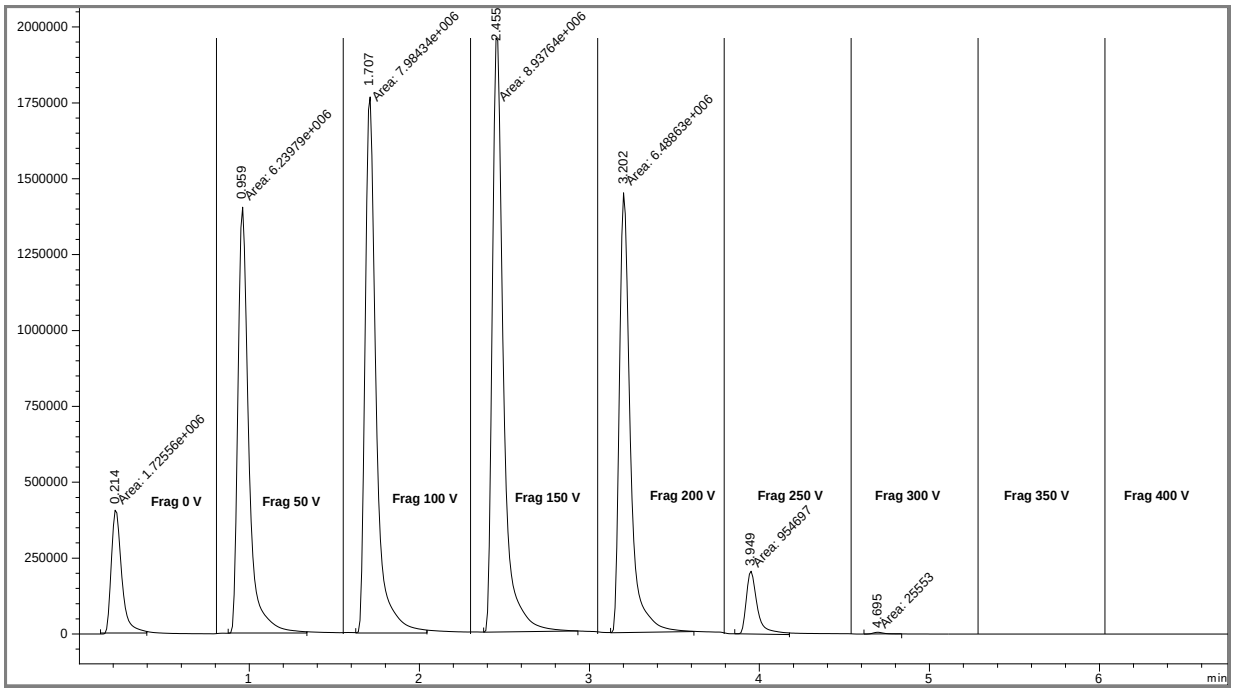



**Figure 3.3:** **FIA on fragmentor voltage through SIM on pseudomolecular ion of standard solution 8-PN (1 mg/mL; 0,1 µL injection).**

Gain values and MS cycle times were individually defined per MS signal to obtain maximum sensitivity. Moreover, two time groups were assigned to the first of four MSD signals, in order to benefit the detection (*i.e.* 100% cycle time) of the hop-derived compounds XN, IX and 8-PN, which elute at the end of the solvent gradient. The HPLC effluent entered the ionization chamber only in the time window between 3.5 and 14.0 min (Table 3.1).

**Table 3.1:** MSD signal groups with target ion values (*m/z*), gain values, fragmentor voltages and cycle times.

| MSD signals | Time windows & Analytes | Ions (*m/z*) | Gain value | Fragmentor voltage (V) | Cycle Time (%) |
|---|---|---|---|---|---|
| **MSD 1** | 3.5-10.4 min | | 1.0 | | 10.0 |
| **(APCI +)** | IS | 199 | | 125 | |
| | DHD | 257 | | 175 | |
| | DAID | 255 | | 125 | |
| | GEN | 271 | | 200 | |
| | 10.41-14.0 min | | 2.0 | | 100.0 |
| | 8-PN | 341 | | 150 | |
| | IX, XN | 355 | | 200 | |
| **MSD 2** | 3.5-10.4 min | | 3.0 | | 30.0 |
| **(APCI +)** | EQ | 243 | | 100 | |
| | *O*-DMA | 259 | | 125 | |
| | COUM | 269 | | 175 | |
| **MSD 3** | 3.5-10.4 min | | 3.0 | | 30.0 |
| **(APCI +)** | SECO | 327 | | 120 | |
| | MAT | 359 | | 125 | |
| **MSD 4** | 3.5-10.4 min | | 3.0 | | 30.0 |
| **(APCI -)** | ENL | 297 | | 125 | |
| | END | 301 | | 175 | |

In Figure 3.4, merged, normalized MS chromatograms of the standard mixture in solvent are illustrated at medium level of the calibration range.

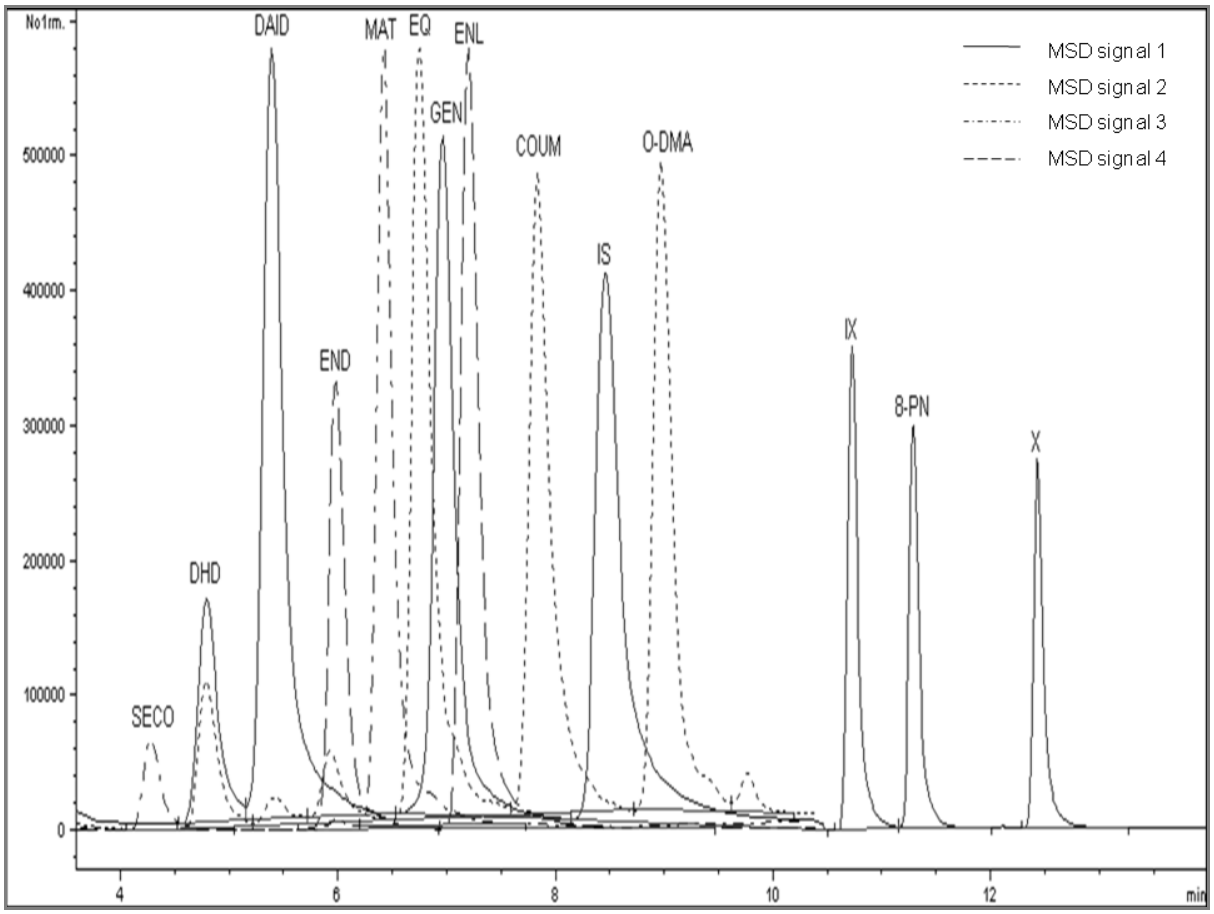



**Figure 3.4:** **Normalized and merged MS chromatograms of the four MSD signals (pure standard mixture at medium range of calibration). Note: the appearance of some peaks in multiple signals are due to overlay of the window of *m/z* values and/or small impurities in the standards; peak tailing is due to the solvent strength of the injection solvent (100% MeOH).**

## *3.2 Method validation results*

- **Selectivity**

Only a few small peaks were observed in the mass chromatograms of the different blank and zero urine and serum samples (resolution between target and interfering peak always between 0.5 and 1.0). Since the present method also makes use of extracted ions for quantification, the background is further reduced and the sensitivity as well as specificity are increased. Moreover, simultaneous recording of characteristic retention times and UV spectra favoured accurate identification and quantitation of the target compounds. During validation, appropriate blanks were included to correct for any possible interference problems.

- **Assessment of linearity and sensitivity**

The linearity was tested in urine for the range of concentrations 0.1-400.0 ng/mL for IX, XN and COUM and 1.0 to 4000.0 µg/mL for DAID, GEN, DHD, *O*-DMA, EQ, SECO, MAT, END and ENL. In serum, the concentrations ranged from 0.04 to 100.0 ng/mL for IX, XN and COUM, and 0.4 to 1000.0 ng/mL for the other compounds. Calibration curves showed linear responses for all analytes over the dynamic ranges and the corresponding regression correlation coefficients ($r^2$) were all > 0.995. Weighting was not considered since the linearity of all thirteen compounds in both urine and serum was satisfactory. Indeed, visual inspection of residual plots confirmed the hypothesis of homogeneity of variance (randomly scattered). In addition, the residuals were in accordance with the stated acceptance criteria, as the %RRE (% relative residual error, *i.e.* percent relative difference of the residual from the nominal value at a certain calibration point) was for all standards ≤ 15% at each calibration point. The limits of detection (S/N=3) for all compounds, both in urine and serum, are listed in Table 3.2. In urine, the LODs ranged from 0.2 to 7.7 ng/mL, and in serum, from 1.4 to 20.4 ng/mL, except for SECO which gave LODs in urine and serum of 65.1 ng/mL and 132.6 ng/mL, respectively. If necessary, the target compounds, at the

levels which are assumed to be below the corresponding LOD in real samples, can be reanalysed with only one MSD signal active to further improve the sensitivity of detection. The limits of quantification were set as the lowest point of the calibration curves with a signal-to-noise ratio of at least 10.

**Table 3. 2:** Limits of detection (LOD) in urine and serum.

| Limit of detection (LOD) | | | | |
|---|---|---|---|---|
| | (ng/mL) | | (nmol/L) | |
| Compound | Urine | Serum | Urine | Serum |
| **Dihydrodaidzein** | 2.4 | 7.9 | 9.4 | 31.1 |
| **Daidzein** | 1.0 | 1.9 | 3.9 | 7.4 |
| **Genistein** | 0.8 | 2.2 | 3.0 | 8.1 |
| **Isoxanthohumol** | 0.2 | 1.5 | 0.5 | 4.2 |
| **8-Prenylnaringenin** | 0.4 | 1.5 | 1.2 | 4.4 |
| **Xanthohumol** | 0.6 | 1.4 | 1.7 | 4.0 |
| **Enterodiol** | 2.7 | 3.1 | 8.9 | 10.3 |
| **Enterolactone** | 2.5 | 2.4 | 8.4 | 8.1 |
| **Equol** | 5.8 | 13.8 | 24.0 | 57.0 |
| **Coumestrol** | 0.4 | 6.2 | 1.5 | 23.1 |
| ***O*-DMA** | 7.7 | 20.4 | 29.8 | 79.1 |
| **Secoisolariciresinol** | 65.1 | 132.6 | 179.8 | 366.3 |
| **Matairesinol** | 7.5 | 17.8 | 20.9 | 49.7 |

- **Accuracy and precision**

The accuracy and precision of the method were determined by spiking blank urine and serum samples at low, medium and high quality control levels, with six replicates on four consecutive days. The accuracy, expressed as the percentage relative error (%RE), was

within the acceptable ranges of 20% for all compounds at all concentrations. In urine, the accuracy ranged from 0.2% to 14.6% and, in serum, from 0.01% to 18.7% (Table 3.3).

The precision of the method is reflected in the variance of quality control samples over time. Inter- and intra-assay variations in urine and serum were thoroughly investigated for the three QC-levels and were expressed as %CV (Table 3.4). The %CV for the inter-assay precision in urine and serum did not exceed the acceptable 20% and ranged from 4.3% to 18.8% and 4.4% and 15.7% in urine and serum, respectively. The intra-assay precision in urine was overall better than 10% (%CV < 15% as required by the acceptance criteria), except for END, for which, at medium level was 14.0%. In serum, the intra-assay precision results were also conform with the stated criteria and with the exception of DAID and END, at respectively high and low QC-level, were also below 10%.

**Table 3.3:** **Accuracy (%) in urine and serum for three different QC levels (*n*=6).**

| | Urine | | | | Serum | | |
|---|---|---|---|---|---|---|---|
| | Low QC | Medium QC | High QC | | Low QC | Medium QC | High QC |
| **DHD** | 4.7 | 0.5 | 0.7 | | 3.8 | 6.3 | 0.4 |
| **DAID** | 1.6 | 10.3 | 11.4 | | 4.0 | 0.7 | 4.9 |
| **GEN** | 1.8 | 13.1 | 13.4 | | 3.1 | 0.08 | 7.5 |
| **IX** | 7.1 | 2.6 | 0.2 | | 11.8 | 14.0 | 6.0 |
| **8-PN** | 10.5 | 11.1 | 8.6 | | 6.6 | 6.4 | 9.2 |
| **XN** | 12.0 | 4.5 | 14.6 | | 6.4 | 9.0 | 0.01 |
| **END** | 6.9 | 5.2 | 8.5 | | 15.0 | 8.3 | 16.7 |
| **ENL** | 11.0 | 5.6 | 7.3 | | 17.3 | 4.6 | 14.4 |
| **EQ** | 2.7 | 1.1 | 1.4 | | 1.7 | 6.7 | 6.5 |
| **COUM** | 0.9 | 0.7 | 1.5 | | 14.4 | 3.0 | 18.7 |
| ***O*-DMA** | 5.2 | 9.2 | 3.2 | | 8.2 | 12.7 | 8.4 |
| **SECO** | 10.4 | 6.2 | 0.2 | | 14.8 | 4.9 | 4.5 |
| **MAT** | 4.7 | 0.2 | 6.9 | | 12.9 | 12.8 | 14.5 |

**Table 3.4:** Inter- and intra-assay precision (%CV) for three different QC levels (*n*=6) in urine and serum.

| | Inter-assay (urine/serum) | | | Intra-assay (urine/serum) | | |
|---|---|---|---|---|---|---|
| | Low QC | Medium QC | High QC | Low QC | Medium QC | High QC |
| **DHD** | 14.3 / 13.4 | 10.8 / 7.6 | 14.6 / 11.5 | 6.9 / 2.9 | 6.6 / 6.8 | 5.6 / 5.9 |
| **DAID** | 17.8 / 12.8 | 10.5 / 6.1 | 14.4 / 11.3 | 6.9 / 9.1 | 5.0 / 5.1 | 6.4 / 10.8 |
| **GEN** | 11.7 / 13.7 | 8.4 / 5.3 | 13.0 / 13.2 | 7.4 / 3.0 | 5.0 / 3.7 | 4.1 / 5.6 |
| **IX** | 18.7 / 11.1 | 9.2 / 15.0 | 4.3 / 11.2 | 7.2 / 3.3 | 5.4 / 5.7 | 3.1 / 7.9 |
| **8-PN** | 15.6 / 11.2 | 12.6 / 7.5 | 15.0 / 10.4 | 8.7 / 3.1 | 6.1 / 1.2 | 3.3 / 7.4 |
| **XN** | 16.0 / 10.5 | 15.3 / 7.8 | 17.6 / 11.9 | 9.3 / 3.4 | 8.1 / 3.7 | 3.8 / 8.4 |
| **END** | 10.7 / 11.2 | 15.8 / 5.2 | 10.9 / 6.5 | 8.1 / 10.7 | 14.0 / 7.8 | 5.4 / 6.4 |
| **ENL** | 14.0 / 10.5 | 14.4 / 7.8 | 10.4 / 15.4 | 9.4 / 3.2 | 5.6 / 3.2 | 4.5 / 3.5 |
| **EQ** | 14.3 / 11.0 | 15.2 / 7.5 | 12.1 / 13.4 | 5.6 / 3.3 | 6.5 / 1.4 | 8.2 / 6.8 |
| **COUM** | 13.3 / 13.1 | 16.8 / 4.4 | 15.1 / 14.1 | 7.3 / 9.3 | 5.1 / 1.3 | 3.3 / 10.0 |
| ***O*-DMA** | 18.8 / 10.0 | 17.8 / 6.4 | 6.6 / 12.5 | 6.0 / 3.0 | 10.0 / 5.2 | 5.7 / 5.8 |
| **SECO** | 14.8 / 15.7 | 13.4 / 11.9 | 16.5 / 15.5 | 7.4 / 2.8 | 3.8 / 5.1 | 6.9 / 8.4 |
| **MAT** | 12.3 / 13.4 | 14.7 / 7.1 | 17.6 / 9.5 | 7.1 / 2.9 | 4.1 / 6.2 | 7.0 / 7.5 |

- **Stability**

The chemical stability of the analytes was assessed in the target matrix under specific conditions of time and temperature. In particular, the short-term stability was investigated in urine and serum. In urine, all components were stable in the processed samples after a period of 15 h in the autosampler (room temperature), except for END and ENL. At both low and high levels a significant ($P < 0.05$) and consistent increase in concentration was found for END and ENL (*i.e.* 54.5% increase at low and 37.8% increase at high level for END and 54.0% at low and 31.5% at high level for ENL). With the exception of a significant ($P < 0.05$) average decrease of 26.1% in concentration of COUM at low QC level, the stability of processed serum samples after 15 h in the autosampler was confirmed. Furthermore, in urine, following three repeated cycles of freezing and thawing, only IX and 8-PN showed degradation in the low QC samples (*i.e.* > 20% difference in concentration between reference and stability samples). At both low and high levels, an acceptable but slight degradation was noticed for END and ENL (> 15% and < 20% difference). Finally, an instability of MAT, estimated as an average of 42.0% (± 4.8%) loss in concentration of MAT at low and high levels was observed after three freeze and thaw cycles.

- **Recovery and matrix effect**

The recoveries (IS-normalized) in urine for all analytes were greater than 67%, both for low and high concentrations. In serum samples, the average recoveries were found to be higher than 83% for most analytes, except for IX, XN and COUM. The lower recoveries in serum for IX and XN (*i.e.* 40.6% and 46.8% for IX and 69.1% and 58.8% for XN at low and high level, respectively) and especially for COUM, which had an average recovery of 24.1% (± 5.0%), can be explained by strong retention on the SPE sorbent due to their relatively non-polar character and the low solubility of COUM in the elution solvent (MeOH). All recoveries at high level are listed in Table 3.5.

**Table 3.5:** Absolute recovery (%) in urine and serum at high QC level (*n*= 6) (%RSD).

| | Urine | Serum |
|---|---|---|
| **DHD** | 97.3 (14.4) | 89.2 (7.8) |
| **DAID** | 119.0 (11.4) | 95.2 (6.2) |
| **GEN** | 99.6 (10.6) | 90.6 (8.5) |
| **IX** | 91.9 (16.9) | 46.8 (9.4) |
| **8-PN** | 78.7 (14.9) | 88.3 (12.2) |
| **XN** | 74.1 (16.1) | 58.8 (16.1) |
| **END** | 99.8 (9.7) | 95.6 (9.4) |
| **ENL** | 115.5 (10.1) | 95.0 (7.1) |
| **EQ** | 91.1 (11.9) | 95.5 (6.2) |
| **COUM** | 89.7 (16.4) | 19.7 (14.0) |
| ***O*-DMA** | 91.7 (15.2) | 83.2 (9.3) |
| **SECO** | 67.5 (15.9) | 95.8 (7.8) |
| **MAT** | 87.8 (14.6) | 98.0 (7.2) |

In this study, matrix effects (ME%) in urine and serum were evaluated by analyzing low and high QC samples (only the matrix effects of the high QC samples in urine and serum are shown here in Table 3.6). In urine, an overall ion enhancement was observed, especially for GEN and COUM, with an average of 43% and 49% increase of the signal due to matrix effects, respectively. Serum matrix components resulted for SECO in more than a twofold increase of the observed signal. The high matrix effect observed with SECO may be attributed to co-elution in the beginning of the gradient with retained matrix components that lead to a strong enhancement of the ionization of SECO and subsequent overestimation of measured concentrations.

**Table 3.6:** Matrix effects (ME% (RSD%)) in urine at high QC level in 6 different matrix blanks (*n*=3).

| | Urine | | | | | |
|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **E** | **F** |
| **DHD** | 95.2(7) | 111.9(4) | 111.3(6) | 113.8(7) | 104.0(7) | 106.6(3) |
| **DAID** | 104.9(8) | 115.5(13) | 111.3(7) | 117.7(10) | 134.0(3) | 132.4(1) |
| **GEN** | 131.0(13) | 136.0(6) | 146.9(9) | 159.8(14) | 150.7(7) | 133.4(3) |
| **IX** | 109.7(3) | 116.6(5) | 125.1(4) | 148.6(3) | 107.8(7) | 120.5(6) |
| **8-PN** | 112.4(7) | 115.8(5) | 109.0(2) | 120.3(15) | 102.7(6) | 105.2(2) |
| **XN** | 107.5(12) | 108.8(10) | 91.2(3) | 102.2(6) | 85.6(8) | 82.5(6) |
| **END** | 98.1(11) | 123.6(4) | 127.2(3) | 124.9(5) | 101.1(7) | 85.5(6) |
| **ENL** | 86.6(11) | 106.7(4) | 110.3(7) | 117.9(5) | 101.3(6) | 66.6(8) |
| **EQ** | 86.6(10) | 122.5(9) | 93.6(5) | 111.0(3) | 94.3(12) | 93.3(7) |
| **COUM** | 149.2(13) | 145.4(9) | 165.1(9) | 127.4(12) | 161.6(10) | 147.1(1) |
| ***O*-DMA** | 108.1(6) | 102.2(7) | 99.9(6) | 102.8(8) | 98.8(7) | 103.4(9) |
| **SECO** | 95.6(6) | 128.4(12) | 138.6(11) | 116.8(7) | 105.3(13) | 109.1(5) |
| **MAT** | 99.1(5) | 121.2(8) | 113.3(5) | 127.9(9) | 101.8(6) | 108.2(2) |

A relative overall ion enhancement was noticed in serum (Table 3.7), although more stabilized matrix effects for SECO and the other components were observed in one of the six batches of serum (serum F). In that particular case, the serum was obtained from a totally different source (see section 2.2, purchased pooled human blank serum) and did not represent the average type of serum sample that is generally obtained during the clinical trials this method was developed for.

**Table 3.7:** **Matrix effects (ME% (RSD%)) in serum at high QC level in 6 different matrix blanks (*n*=3).**

| | Serum | | | | | |
|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **E** | **F** |
| **DHD** | 107.3(4) | 116.9(4) | 112.0(5) | 113.0(6) | 101.1(7) | 83.9(6) |
| **DAID** | 113.0(4) | 116.3(3) | 118.4(4) | 120.3(5) | 124.2(3) | 93.3(2) |
| **GEN** | 131.3(5) | 135.6(3) | 136.2(2) | 137.2(5) | 131.7(8) | 93.2(1) |
| **IX** | 119.6(6) | 126.4(1) | 117.4(2) | 123.6(4) | 121.9(7) | 91.2(2) |
| **8-PN** | 104.5(8) | 111.8(7) | 91.1(11) | 103.4(6) | 99.8(1) | 70.9(11) |
| **XN** | 135.8(3) | 136.4(1) | 181.0(8) | 162.8(3) | 139.5(14) | 96.8(3) |
| **END** | 106.8(6) | 131.6(5) | 150.7(3) | 158.8(3) | 133.4(5) | 101.7(2) |
| **ENL** | 98.1(4) | 112.5(1) | 141.3(4) | 144.5(2) | 124.3(6) | 101.6(2) |
| **EQ** | 114.2(8) | 113.5(2) | 108.2(7) | 110.5(7) | 109.4(15) | 88.6(6) |
| **COUM** | 131.4(4) | 132.3(5) | 127.8(3) | 129.3(3) | 125.3(3) | 99.4(4) |
| ***O*-DMA** | 126.6(2) | 123.9(7) | 130.0(3) | 123.5(4) | 113.4(11) | 85.6(4) |
| **SECO** | 253.4(10) | 235.3(9) | 261.9(7) | 281.6(4) | 214.8(3) | 150.2(5) |
| **MAT** | 130.9(9) | 127.4(5) | 119.3(4) | 121.7(8) | 128.7(3) | 92.7(5) |

Moreover, the variation of the overall matrix effect in urine and serum samples was typically less than 15% (urine (A - F): 3.5 to 14.8% and serum (A - E): 2.0 to 15.7%) and considered relatively stable. This observation concerning matrix effects again highlights the need for multiple source relative matrix effect evaluation instead of assessments based on single source biological fluid measurements. As a conclusion, these findings of the matrix effects caused by urine and serum factors have clearly shown the relevance of a profound and extensive investigation during LC/MS method development and validation but were not the initial intention of this project.

## 4. CONCLUSION

This HPLC/APCI-MS methodology represents a new quantitative assay for the simultaneous determination of thirteen phytoestrogens, including their most important precursors and gut microbial metabolites, in human urine and serum. The method applies a relatively short and low-cost sample preparation procedure. Validation parameters revealed that accuracy, inter- and intra-assay precision are within the general ranges for acceptance and furthermore the sensitivity is in the lower ppb-range. Moreover, the detection system can be easily integrated in a standard small-scale analytical laboratory. This validated method was successfully applied for the analyses of various urine and serum samples collected in a dietary intervention trial with food supplements combining different classes of phytoestrogens, as reported in detail in Chapter 4 [130]. The strength of our method, as mentioned above, is the ability to measure a wide panel of analytes in complex biological matrices, even after supplementation of mixtures of phytoestrogen-rich supplements. It can be concluded that the present method, covering the detection of a wide and representative range of dietary phytoestrogens should provide a framework for extensive analysis in a (pre)clinical environment, whenever efficiency studies are impeded by inter-individual differences in exposure levels.

# CHAPTER 4

# Application of a LC-MS method to a dietary intervention study with mixed phytoestrogens

This chapter was published as:

*Cosupplementation of isoflavones, prenylflavonoids, and lignans alters human exposure to phytoestrogen-derived 17β-estradiol equivalents.*

Bolca, S., Wyns, C., Possemiers, S., Depypere, H., De Keukeleire, D., Bracke, M., Verstraete, W. and Heyerick, A.

Journal of Nutrition, 2009. 139(12): p 2293-300.

# 1. INTRODUCTION

Whether mixtures of phytoestrogens at relevant nutritional doses can add to, synergize or antagonize with the endogenous estrogens is currently debated [131-133]. Yet, an important aspect that tends to be overlooked in these multi-exposure experiments is the crucial role of the gut microbiota with regard to the bioavailability and bioactivity of phytoestrogens [134, 135]. In the colon, several microbial transformations generating more bioactive metabolites, can occur. The conversion of daidzein to equol [136], isoxanthohumol (IX) to 8-prenylnaringenin (8-PN) [137], and secoisolariciresinol (SECO) to enterolactone (ENL) [138] results in an approximately 15- [139], 100- [140], and 75- [141] fold increase in estrogenic potency, respectively. Since, unlike most other animals and especially rodents, not all humans are hosting phytoestrogen-metabolizing intestinal micro-organisms [142], the extent of these microbial bioactivations varies considerably among individuals and, consequently, weak, moderate, and strong producer phenotypes can be differentiated [143].

In view of the increasing number of food supplements combining several classes of phytoestrogens, we investigated the microbial potential to activate various proestrogens within an individual, and evaluated the final exposure to microbial metabolites of isoflavones, prenylflavonoids, and lignans upon coadministration in 3 randomized dietary crossovers (Figure 4.1). To the best of our knowledge, this is the first controlled human study addressing these issues.

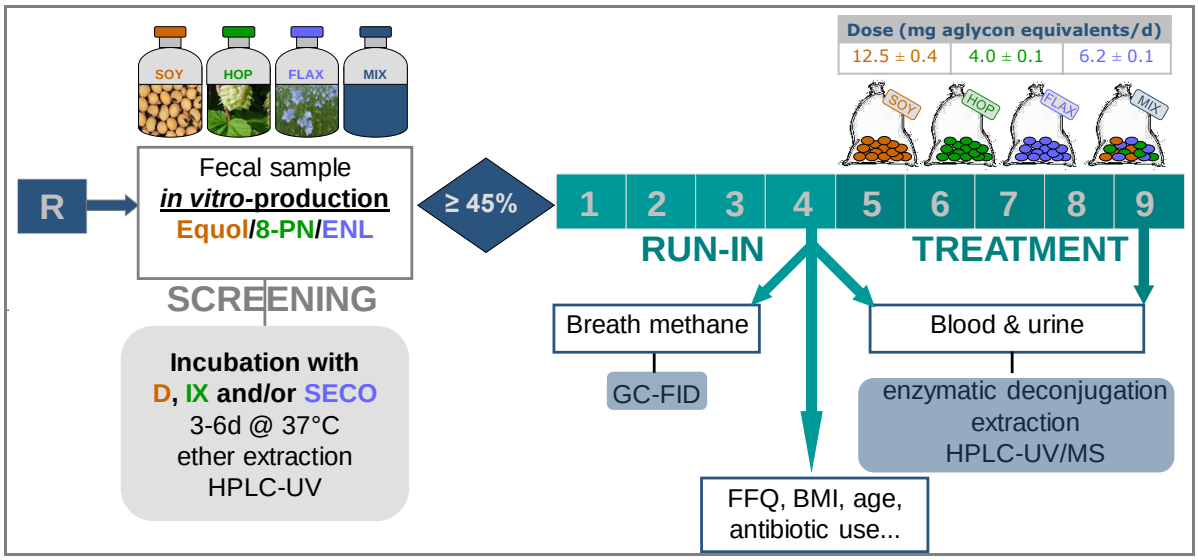



**Figure 4.1:** Study design of a dietary intervention trial, focusing on the screening and the first intervention arm of a crossover. Treatment allocation was based on participants' eligibility (*i.e.*, >45% *in vitro*-bioactivation of ≥2 separate proestrogens by fecal cultures; *n*= 40/100). After a run-in of ≥4 d, participants were given soy, hop, and/or flax-based food supplements, dosed either separately (SOY, HOP or FLAX; reference intervention) or simultaneously (MIX; test intervention), 3 times/d during 5 d, followed by a wash-out (≥7 d) and the second intervention.

# 2. EXPERIMENTAL

## *2.1 Phytoestrogens*

- **Standards and extracts**

Genistein, daidzein, secoisolariciresinol (SECO), matairesinol, enterodiol (END), and ENL were purchased from Sigma-Aldrich (Bornem, Belgium), equol from Extrasynthèse (Genay, France), and dihydrodaidzein (DHD) and *O*-desmethylangolensin (*O*-DMA) from Plantech UK (Reading, UK). Xantho-Flav™, a xanthohumol (XN)-rich extract, was kindly provided by Hopsteiner (Mainburg, Germany) and used for the isomerization of XN into isoxanthohumol (IX) and preparation of 8-prenylnaringenin (8-PN) [144]. SoyLife® EXTRA and LinumLife™ EXTRA, provided by Frutarom Netherlands (Veenendaal, The Netherlands), were used as a source of daidzein and SECO, respectively, in the incubation experiments.

- **Food supplements**

Commercially available food supplements, kindly provided by their manufacturers, were used in this study with following contents: soy-based tablets (Bio-Flavon, Metagenics Europe, Ostend, Belgium): 6.09±0.10 mg genistein and 2.83±0.05 mg daidzein aglycone equivalents per tablet; hop-based capsules (MenoHop®, Metagenics Europe, Ostend, Belgium): 2.04±0.06 mg XN, 1.20±0.04 mg IX, 0.10±0.01 mg 8-PN per capsule; flax-based tablets (Linidol, NutriPhyt, Oostkamp, Belgium): 2.08±0.02 mg SECO aglycone equivalents per tablet.

- **Participants**

Since so far, no studies revealed gender effects as major determinants of phytoestrogen metabolism and kinetics [34, 145], both men (*n*= 30) and women (*n*= 70)

were recruited. A total of 100 generally healthy individuals, living and/or working in the area of Ghent, Belgium, participated in this study. Their age and BMI, based on self-reported weight and height measurements, ranged between 18-78 y and 17-32 kg/m², respectively. The exclusion criteria were the use of antibiotics within the previous month and soy or flax allergy. Ethical approval was granted by the Ethics Committee of the Ghent University Hospital (EC UZG 2007/199; Belgian registration number: B67020072203). The volunteers were fully informed on the aims of the study and gave their written consent.

### *2.2 Study design*

- **Screening**

Participants were asked to deliver a fresh fecal sample to assess their equol, 8-PN, and ENL producer phenotypes. Within 6 h after collection, fecal suspensions were prepared and incubated (10/90, v/v) anaerobically at 37 °C in Brain Heart Infusion broth (37 g/L, Oxoid, Erembodegem-Aalst, Belgium) supplemented with L-cystein·HCl (0.5 g/L), resazurin (2 mg/L), and a source of either daidzein (529.8±5.3 µmol/L daidzein aglycone equivalents as SoyLife® EXTRA, Frutarom Netherlands, Veenendaal, The Netherlands; 'SOY') or IX (61.2±0.2 µmol/L IX, 'HOP') or SECO (645.8±20.1 µmol/L SECO aglycone equivalents as LinumLife™ EXTRA, Frutarom Netherlands, Veenendaal, The Netherlands; 'FLAX'), as described by Possemiers *et al.* [143]. After 3 and 6 d, samples were taken to analyze the daidzein and IX metabolism, and the SECO metabolism, respectively. This phenotyping based on the phytoestrogen metabolism by fecal cultures, proved to be valid for equol [144] and 8-PN [31].

At the same time, the microbial cultures were additionally incubated with a mixture of daidzein glycoside, IX, and SECO-diglucoside (529.8±5.3 µmol/L daidzein aglycone equivalents + 61.2±0.2 µmol/L IX + 645.8±20.1 µmol/L SECO aglycone equivalents, 'MIX') to investigate the ability to metabolize simultaneously isoflavones, prenylflavonoids, and lignans *in vitro*.

- **Run-in and wash-out phases**

Only 'multi-producers', defined as individuals hosting intestinal microbiota bioactivating >45% of 2 or more separately supplemented precursors *in vitro* (*n*= 40/100), were included in the dietary intervention trials. These consisted of a run-in phase of at least 4 d, a first intervention with phytoestrogens (test or reference) during 5 d, a wash-out phase of at least 7 d, and a second intervention with phytoestrogens (test or reference) during 5 d. The participants were counseled not to change their habitual, Western-type dietary patterns, but were asked to abstain from soy, hop, and flax-derived products in order to minimize interferences from other dietary sources of isoflavones, prenylflavonoids or lignans. A detailed list of phytoestrogen-containing foods and dietary supplements was distributed in order to guide the volunteers in this respect. Additionally, participants were instructed to report in a diary every case of doubt or fortuitous consumption, and to provide detailed information on that eating occasion, including type and portion size.

- **Dietary interventions**

The dietary intervention trials were randomized crossovers. As reference treatment, a food supplement containing either isoflavones ('SOY') or prenylflavonoids ('HOP') or lignans ('FLAX') was dosed, whereas the test treatment consisted of coadministration of these 3 supplements as one dosage ('MIX'). During each intervention phase (5 d), food supplements were taken daily with meals at breakfast, lunch, and dinner (3 dosages/d). Following the eligibility assessment, 40 volunteers were invited to the dietary intervention trials, but 3 withdrew. Thirty-seven were allocated to the SOY+MIX (*n*= 22), HOP+MIX (*n*= 21) or FLAX+MIX (*n*= 21) interventions according to their *in vitro*-producer phenotypes, *e.g.,* an equol+8-PN producer was randomly assigned to either SOY+MIX or HOP+MIX or both. In total, 35 individuals (10 men, 16 premenopausal and 9 postmenopausal women) completed the study: 22 equol producers, 19 8-PN producers, and 21 ENL producers participated in the SOY+MIX, HOP+MIX, and FLAX+MIX trials, respectively. Compliance was evaluated by participant inquiry, remaining supplement counts, and urinary phytoestrogen

excretion. The volunteers were also asked to record when they took the food supplements or, in case, forgot them.

- **Sample collection**

After the run-in and wash-out phases, and on the last day of each intervention, spot urine and blood samples were collected together with the diaries. Timing of the venipunctures and urine collections was standardized for each individual and ranged between 0.5-5.0 h after the first supplement intake of that day (breakfast). Although steady-state levels are reached after 5 d of regular intake throughout the day [147, 148], diurnal fluctuations are expected, especially for the plant precursors, because of the discontinued dosing during night, as shown for daidzein and genistein [149]. At the study onset, however, no information was available on an optimal time of sampling for isoflavones, prenylflavonoids, lignans, and their microbial metabolites. Therefore, the sampling interval was chosen based on feasibility aspects, such as participant burden. Serum was obtained by centrifugation (10 min, 600 *g*, room temperature (RT)) after coagulation. Aliquots of both urine and serum samples were stored at -20 °C until analysis.

In addition, a general questionnaire was used to get information on the participants' history of antibiotic treatments, hormonal therapies, use of any other medication, dietary habits and food supplement intakes, anthropometric measures, and physical activities.

### *2.3 Analytical methods*

- **Enzymatic hydrolysis (urine and serum)**

Total, *i.e.,* free and conjugated, phytoestrogens in the urine and serum samples were extracted upon enzymatic hydrolysis with a Type H-1 *Helix pomatia* extract (Sigma Aldrich, Bornem, Belgium) in sodium acetate buffer (0.1 mol/L, pH= 5), using a protocol validated in

Chapter 3 [150]. The urine samples (2 mL) were incubated with 30 U β-glucuronidase and 1.53 U sulfatase for 1 h at 37 °C, whereas the serum samples (800 µL) were spiked with 15 µL internal standard (0.4 mmol/L 4-hydroxybenzophenone in methanol) and defatted with hexane (50/50, v/v) prior to incubation with 7380 U β-glucuronidase and 376 U sulfatase for 24 h at 37 °C.

- **Liquid-liquid extraction (fecal incubation broths and urine)**

The protocols described by Bolca *et al.* [146] were adapted to extract isoflavones, prenylflavonoids, and lignans simultaneously. Each sample (fecal incubations: 1 mL; urine: 2 mL) was mixed (50/50, v/v) with sodium acetate buffer (0.1 mol/L, pH= 5), spiked (fecal incubations: 50 µL, urine: 25 µL) with internal standard (0.4 mmol/L 4-hydroxybenzophenone in ethyl acetate) and extracted twice with 5 mL diethyl ether. The combined extracts were evaporated to dryness at RT under a gentle stream of $N_2$, reconstituted in the mobile phase (fecal incubations: 200 µL, urine: 100 µL), and stored at -20 °C prior to HPLC-UV (fecal incubations) or HPLC-UV/MS (urine) analysis.

- **Solid-phase extraction (serum)**

Serum phytoestrogens were measured after enzymatic hydrolysis and solid-phase extraction (SPE), as validated in Chapter 3 [150]. The SPE-cartridges (Bond Elut® $C_{18}$, 3 mL, 200 mg, Varian, Sint-Katelijne-Waver, Belgium) were activated with 3 mL methanol and conditioned with 3 mL sodium acetate buffer (0.1 mol/L, pH= 5), consecutively. After sample application, the cartridges were rinsed with 3 mL methanol/water (5/95, v/v) and the compounds of interest were eluted with 3 mL methanol using a VacMaster 20 sample processing unit (IST, Hengoed, Mid Glamorgan, UK). The solvent was evaporated to dryness at RT under a gentle stream of $N_2$, reconstituted in 80 µL of the mobile phase, and stored at -20 °C prior to HPLC-UV/MS analysis.

- **Creatinine measurement (urine)**

To allow standardization of diuresis, the urinary excretion of creatinine was measured based on a modified protocol of Hall *et al.* [151]: upon incubation of 100 µL urine with 1.5 mL alkaline picrate reagent (*i.e.,* a 57 mmol/L-picric acid solution (Sigma Aldrich, Bornem, Belgium) mixed with 8.8 g/L NaOH, 19.0 g/L $Na_2HPO_4$, and 19.0 g/L $Na_2B_4O_7.10\ H_2O$ in deionized water, 1:1 (v:v), pH 12.6) during 30 min at 37 °C, 100 µL acetic acid (5.2 mol/L) was added and incubated again for 5 min at 37 °C. The absorbance at 540 nm was measured (Tecan Sunrise™, Tecan Benelux, Mechelen, Belgium) before and after acidification and used to calculate the creatinine concentrations. Daily urinary phytoestrogen excretions were calculated according to the conventional kinetic Jaffé method [152] based on creatinine clearance rates of 0.187 mmol/(d·kg) for men and 0.163 mmol/(d·kg) for women [153].

- **HPLC-UV analysis (fecal incubation broths)**

Quantitative analyses of phytoestrogens and their microbial metabolites (isoflavones: genistein, daidzein, DHD, *O*-DMA, and equol; prenylflavonoids: IX and 8-PN; and lignans: SECO, END, and ENL) were performed by HPLC-UV using a Waters 2695 Alliance separations module and 996 PDA (Waters, Milford, MA, USA), combined with an XBridge™ $C_{18}$ reversed-phase column (5 µm, 4.6x250 mm; Waters, Milford, MA, USA) and using a gradient of solvent A (*i.e.,* 6.6 mmol/L aqueous formic acid) and solvent B (*i.e.,* 6.6 mmol/L formic acid in acetonitrile/methanol, 20/80 (v/v)) with the following profile: 0-4 min, 40% B in A; 4-6 min, from 40% to 45% B in A; 6-21 min, 45% B in A; 21-25 min, from 45% to 87% B in A; 25-27 min, from 87% to 99% B in A, 27-30 min, from 99% to 40% B in A; 30-35 min, 40% B in A; and a flow rate of 1 mL/min. The injection volume was 20 µL and the column temperature was set at 40 °C. UV detection was done simultaneously at 280 nm for the isoflavones and lignans, and at 295 nm for the prenylflavonoids and the internal standard.

- **HPLC-UV/MS analysis (urine and serum)**

The developed and validated HPLC-UV/MS method as described in Chapter 3 was used for the analysis of urine and serum samples.

- **Statistical analyses**

All extractions were performed in triplicate. SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA) was used to carry out all statistical analyses. Results were considered statistically significant at an α two-tailed level of 0.05. Tests for normality and equality of the variances were performed using the Kolmogorov-Smirnov and Levene's test, respectively. Means and SEM of metabolite formation, and serum and urine concentrations, and differences (Δ) between mean values obtained after separate ('SOY', 'HOP', 'FLAX', reference method) and simultaneous ('MIX', test method) supplementation were calculated. The paired Student's *t*-test or the Wilcoxon's matched-pairs signed-rank test was used to determine significant differences between means. Associations (*r*) were described using Pearson's correlation coefficients or non-parametric Spearman's correlations. Bland & Altman-plots visualized the agreement between the test and reference methods for each compound under investigation at an individual level [154]. Participants were separated according to the bioactivation potential of their fecal cultures (*i.e.,* equol/(daidzein+DHD+equol+*O*-DMA) [144], 8-PN/(IX+8-PN) [31], and ENL/(SECO+END+ENL)) into significantly different groups using the TwoStep cluster protocol. Cross-classification analyses and κ-statistics were applied to evaluate the level of agreement between the producer phenotypes based on the *in vitro*-microbial metabolism of separate *vs.* mixed phytoestrogens.

Power calculations [153] based on an α level of 0.05 and β of 0.05, showed that, with a sample size of 18 participants, differences between the separate and mixed supplementations exceeding 1.25 times the intra-individual variations, would be detectable without generating statistically significant, but scientifically meaningless differences.

# 3. RESULTS

## *3.1 Phytoestrogen metabolism*

As expected, the *in vitro*-microbial metabolism of isoflavones, prenylflavonoids, and lignans varied from 0% up to 100% conversion of daidzein into equol ('SOY' incubations), IX into 8-PN ('HOP' incubations), and SECO into ENL ('FLAX' incubations). Consequently, participants were phenotyped as poor (*n*= 65, 68, and 34), moderate (*n*= 7, 14, and 21), and strong (*n*= 28, 18, and 45) *in vitro*-equol, 8-PN, and ENL producers, respectively. Since the average bioactivation of the moderate producers (mean±SEM: 60±1.5% equol, 64±1.3% 8-PN, and 52±2.1% ENL) exceeded the threshold value of 45%, we merged the moderate and strong producer phenotypes for further analyses. In this way, fecal cultures bioactivating none (*n*= 19), only one (*n*= 41: 12 equol, 7 8-PN, and 22 ENL producers), two (*n*= 33: 0 equol+8-PN producers, 16 equol+ENL producers, and 17 8-PN+ENL producers) or all three (*n*= 7 equol+8-PN+ENL producers) classes of phytoestrogens were characterized (Figure 4.2 A).

However, these bioactivation profiles changed substantially upon incubation with a mixture of isoflavones, prenylflavonoids, and lignans ('MIX' incubations): 64%, 28%, 7%, and 1% of the fecal cultures converted none, only one (*n*= 23 equol, 4 8-PN, and 1 ENL producers), two (*n*= 2 equol+8-PN producers, 2 equol+ENL producers, and 3 8-PN+ENL producers) or all three precursors (Figure 4.2 B). In general, compared to the reference, *i.e.,* separate incubations, the mixed ones yielded lower equol (mean±SEM Δ= 4.4±2.0%, *P*= 0.164), 8-PN (mean±SEM Δ= 20.5±3.9%, *P*< 0.001), and ENL (mean±SEM Δ= 44.3±4.3%, *P*< 0.001) formations. Despite this underestimation, statistically significant correlations were found between the separate and mixed incubations for equol (*r*= 0.925, *P*< 0.001) and 8-PN (*r*= 0.309, *P*= 0.002) formations, but not for ENL (*r*= 0.116, *P*= 0.259). In line with these findings, the homogenous scattering in Figure 4.3 A showed that good estimations were obtained for the equol formation, although some outliners widened the limits of agreement, whereas the Bland & Altman-plots of 8-PN and ENL (Figure 4.3 B and C)

diverged strongly and indicated a high degree of underestimation and considerable variation. Cross-classification revealed a misclassification of 7% (κ= 0.84, *P*< 0.001), 31% (κ= 0.11, *P*= 0.171), and 60% (κ= 0.02, *P*= 0.594), respectively, for the equol, 8-PN, and ENL producer phenotyping based on the mixed incubations. The eligibility assessment of the participants was therefore based on the reference incubations.

To evaluate the biological relevance of these *in vitro* results, the percentages equol, 8-PN, and ENL in urine and serum after administration of a specific type of food supplement ('SOY', 'HOP', 'FLAX' interventions) were compared to those obtained after coadministration ('MIX' interventions). Analyses of the urinary data revealed similar results: a robust equol (mean±SEM Δ= -1.3±1.4%, *P*= 0.149; *r*= 0.651, *P*= 0.001; Figure 4.3 D), a more variable 8-PN (mean±SEM Δ= 1.3±0.5%, *P*= 0.101; *r*= 0.229, *P*= 0.346; Figure 4.3 E), and a highly affected ENL (mean±SEM Δ= 64.2±3.2%, *P*< 0.001; *r*= -0.694, *P*< 0.001; Figure 4.3 F) formation. Additionally, the serum-derived daidzein and IX bioactivation profiles were in line with the *in vitro*-findings (equol: mean±SEM Δ= 5.3±1.2%, *P*= 0.040; *r*= 0.455, *P*= 0.033; Figure 4.3 G; 8-PN: mean±SEM Δ= 1.5±2.5%, *P*= 0.634; *r*= 0.431, *P*= 0.074; Figure 4.3 H). In contrast, the percentages ENL circulating in serum after the FLAX and MIX interventions were similar (mean±SEM Δ= -3.3±0.7%, *P*= 0.030; *r*= 0.694, *P*< 0.001; Figure 4.3 I).

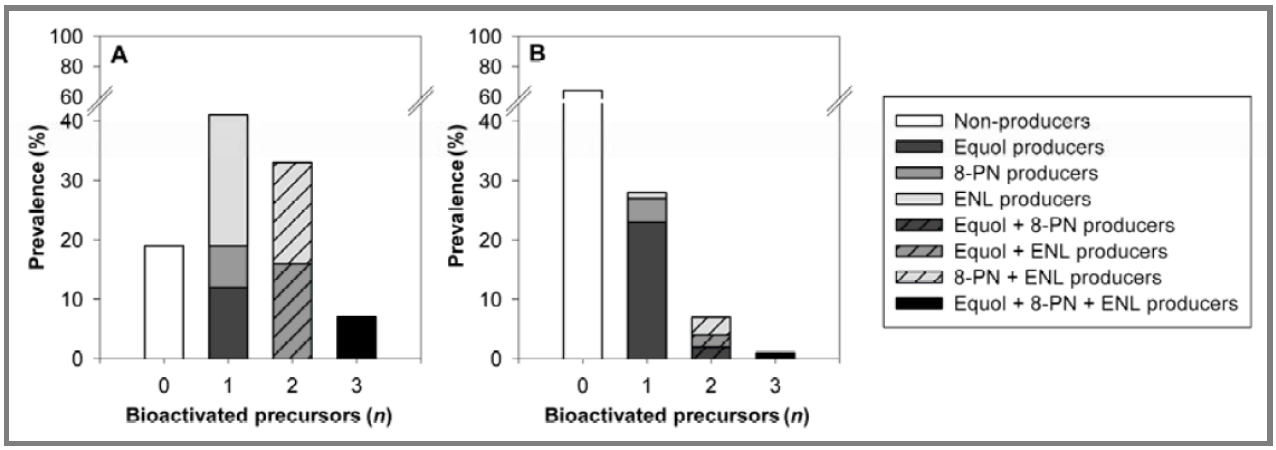



**Figure 4.2:** Prevalence of different *in vitro*-phenotypes based on the equol, 8-PN, and ENL formation by fecal cultures after incubation with a source of daidzein, IX, and SECO (A) separately and (B) simultaneously (*n*= 100).

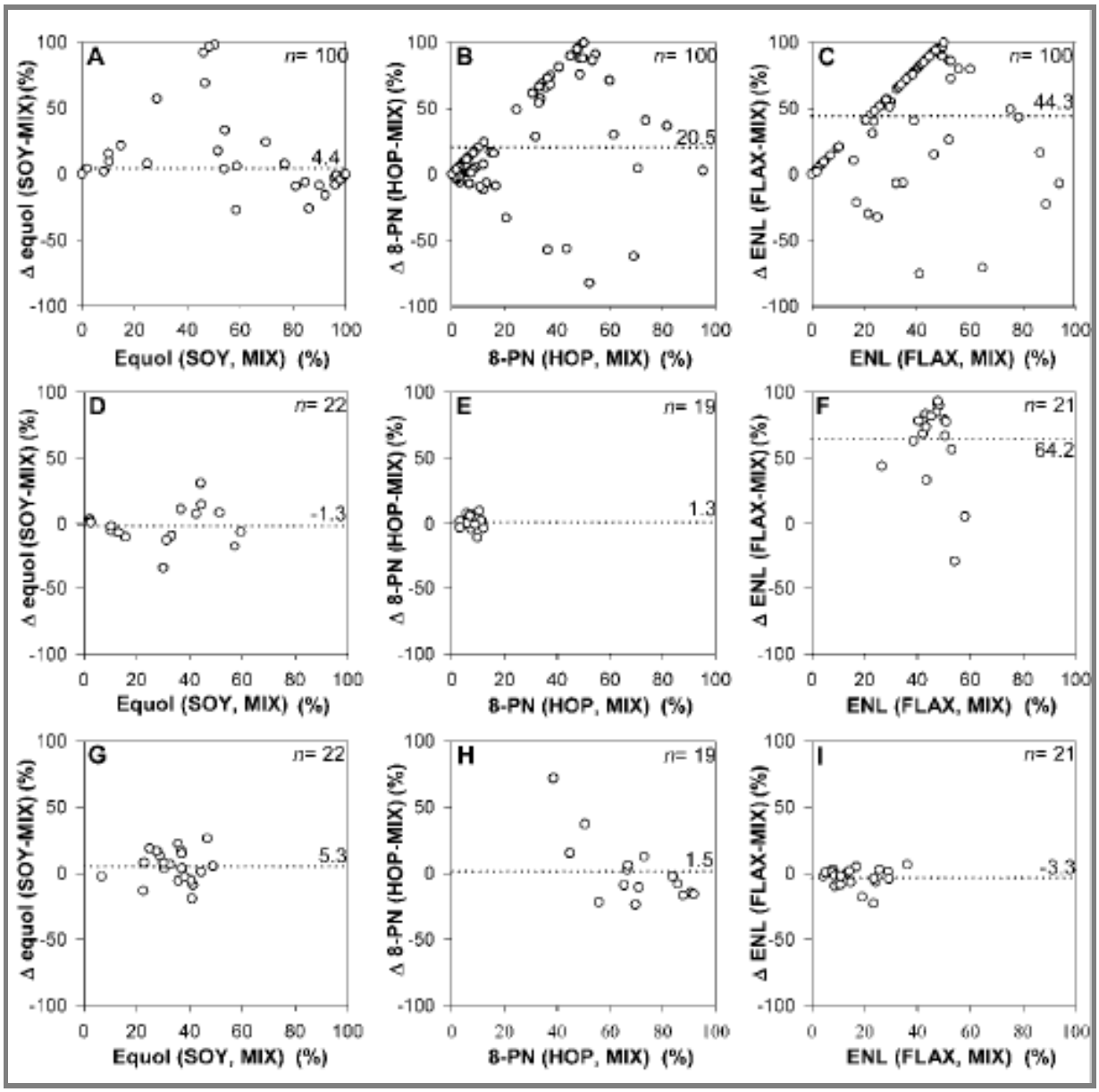



**Figure 4.3:** **Bland & Altman-plots visualizing the differences between the percentages of (A, D, G) equol, (B, E, H) 8-PN, and (C, F, I) ENL (A-C) produced by fecal cultures, (D-F) excreted in urine, and (G-I) circulating in serum after supplementation of a source of daidzein, IX, and SECO separately and simultaneously. The dotted lines represent the mean differences, *n* is indicated on each panel.**

### *3.2 Phytoestrogen exposure*

Analogously, absolute exposures to isoflavones, prenylflavonoids, lignans, and their microbial metabolites were assessed as the sum of unconjugated forms and deconjugated (sulfo)glucuronides and sulfates excreted in urine (Table 4.1) or circulating in serum (Table 4.2), and compared between the treatments ('SOY', 'HOP' or 'FLAX' intervention *vs.* 'MIX' intervention). The estimated daily urinary phytoestrogen excretions varied considerably between individuals and were in the low µmol/d-range after intake of isoflavones ('SOY' interventions: 2.21-18.2 µmol daidzein/d, 0.35-10.3 µmol DHD/d, 0.59-30.8 µmol equol/d, <LOD-19.3 µmol *O*-DMA/d, and 1.35-13.6 µmol genistein/d) and lignans ('FLAX' interventions: <LOD-2.53 µmol SECO/d, 0.06-3.80 µmol END/d, and 1.75-13.9 µmol ENL/d), whereas clearance rates of 96.7-2065 nmol IX/d and 14.8-266 nmol 8-PN/d were estimated after the 'HOP' interventions. The serum concentrations were in the nmol/L-range for isoflavones (42.8-488 nmol daidzein/L, 18.7-143 nmol DHD/L, 41.5-540.3 nmol equol/L, <LOD-399 nmol *O*-DMA/L, and 62.6-1051 nmol genistein/L), lignans (3.80-109 nmol SECO/L, 18.6-275 nmol END/L, and 126-469 nmol ENL/L), as well as prenylflavonoids (24.0-186 nmol IX/L and 26.8-162 nmol 8-PN/L) after intake of soy, flax, and hop-derived food supplements, respectively.

No significant differences were observed between the bioavailabilities of isoflavones, prenylflavonoids, and lignans, proxied by the sum of precursor aglycone equivalents quantified in urine and serum, upon separate and simultaneous administration (total daidzein aglycone equivalents in urine: $P$= 0.391, in serum: $P$= 0.074; total IX equivalents in urine: $P$= 0.503, in serum: $P$= 0.104; SECO aglycone equivalents in urine: $P$= 0.928, in serum: $P$= 0.067). However, participants were exposed to generally lower levels of bioactivated molecules (*i.e.,* equol: Δ= 28.3%, $P$= 0.009; 8-PN: Δ= 35.4%, $P$= 0.107; ENL: Δ= 35.9%, $P$= 0.003) after simultaneous administration of isoflavones, prenylflavonoids, and lignans.

**Table 4.1:** **Urinary excretion of isoflavones, prenylflavonoids, lignans, and their microbial metabolites (mean ± SEM) upon intake of soy-, hop-, and flax-based food supplements separately or simultaneously, difference, and correlation between these excretion profiles.**

| | | Excretion (nmol/mmol creatinine) | | Δ | | Correlation[#] | |
|---|---|---|---|---|---|---|---|
| | | SOY/HOP/FLAX | MIX | % | *P*[§] | *r* | *P* |
| Isoflavones (*n*=22) | **Daidzein** | 729.4 ± 94.4 | 741.7 ± 87.3 | -2.9 | 0.918 | 0.163 | 0.531 |
| | **DHD** | 312.5 ± 55.2 | 224.4 ± 35.9 | 26.0 | 0.177 | 0.483 | 0.050 |
| | **Equol** | 488.8 ± 105.8 | 633.9 ± 151.0 | 1.9 | 0.174 | 0.738 | 0.001 |
| | ***O*-DMA** | 264.0 ± 855.5 | 368.2 ± 91.0 | -30.0 | 0.215 | 0.451 | 0.069 |
| | **Genistein** | 355.4 ± 58.7 | 313.7 ± 44.8 | 7.8 | 0.488 | 0.381 | 0.131 |
| Prenylflavonoids (*n*=19) | **IX** | 57.7 ± 8.3 | 52.6 ± 9.5 | 8.7 | 0.624 | 0.361 | 0.155 |
| | **8-PN** | 4.1 ± 0.5 | 3.4 ± 0.7 | 29.3 | 0.113 | 0.397 | 0.115 |
| Lignans (*n*=21) | **SECO** | 46.5 ± 9.3 | 52.0 ± 11.1 | -18.7 | 0.705 | 0.012 | 0.962 |
| | **END** | 110.1 ± 28.7 | 163.9 ± 76.1 | 25.5 | 0.748 | 0.588 | 0.008 |
| | **ENL** | 526.2 ± 55.0 | 567.8 ± 78.7 | 0.7 | 0.665 | 0.034 | 0.891 |

[#]Pearson's correlation coefficient for all phytoestrogens under investigation, except DHD, *O*-DMA, 8-PN, and END (Spearman's correlation coefficients); [§]Paired Student's *t*-test for all phytoestrogens under investigation, except DHD, *O*-DMA, 8-PN, and END (Wilcoxon's matched-pairs signed-rank test).

**Table 4.2:** Serum concentrations of isoflavones, prenylflavonoids, lignans, and their microbial metabolites (mean ± SEM) upon intake of soy-, hop-, and flax-based food supplements separately or simultaneously, difference, and correlation between those concentrations.

| | | Concentration (nmol/L) | | Δ | | Correlation[#] | |
|---|---|---|---|---|---|---|---|
| | | SOY/HOP/FLAX | MIX | % | *P*[§] | *r* | *P* |
| Isoflavones (*n*=22) | **Daidzein** | 189.3 ± 26.6 | 157.5 ± 18.6 | 11.8 | 0.181 | 0.530 | 0.013 |
| | **DHD** | 60.9 ± 8.6 | 72.1 ± 9.7 | -18.7 | 0.156 | 0.664 | 0.001 |
| | **Equol** | 189.5 ± 17.4 | 137.4 ± 12.4 | 28.3 | 0.009 | 0.311 | 0.169 |
| | ***O*-DMA** | 115.7 ± 25.8 | 109.3 ± 16.4 | -14.4 | 0.795 | 0.410 | 0.072 |
| | **Genistein** | 298.4 ± 48.0 | 155.3 ± 18.8 | 54.0 | 0.003 | 0.457 | 0.037 |
| Prenylflavonoids (*n*=19) | **IX** | 52.0 ± 10.2 | 38.1 ± 9.6 | 52.6 | 0.233 | 0.546 | 0.035 |
| | **8-PN** | 73.4 ± 6.9 | 59.6 ± 10.4 | 35.4 | 0.107 | 0.636 | 0.011 |
| Lignans (*n*=21) | **SECO** | 26.5 ± 5.8 | 50.1 ±7.9 | -70.9 | 0.009 | 0.104 | 0.663 |
| | **END** | 77.1 ± 14.0 | 75.3 ± 10.2 | -0.8 | 0.868 | 0.645 | 0.002 |
| | **ENL** | 226.4 ± 18.4 | 149.6 ± 7.4 | 35.9 | 0.003 | -0.429 | 0.059 |

[#]Pearson's correlation coefficient for all phytoestrogens under investigation, except IX, and SECO (Spearman's correlation coefficients); [§]Paired Student's *t*-test for all phytoestrogens under investigation, except IX and SECO (Wilcoxon's matched-pairs signed-rank test).

# 4. DISCUSSION

To the best of our knowledge, human exposure to microbial metabolites of isoflavones, prenylflavonoids, and lignans administered simultaneously, has not been reported previously, although human nutrition [156] and an increasing number of food supplements contain mixtures of various phytoestrogens. The *in vitro*-incubation experiments with fecal cultures showed that the interplay of phytoestrogens alters the microbial bioactivation profiles yielding generally lower formations of equol, 8-PN, and ENL. Equol formation proved to be the most robust process, whereas conversion of SECO to ENL was severely disturbed by coadministration with soy and hop-derived phytoestrogens. Moreover, similar shifts in bioactivation profiles were observed upon coadministration *in vivo*, yet without affecting the exposure to total isoflavones, prenylflavonoids, and lignans significantly.

The microbial metabolism of daidzein, IX, and SECO as separate compounds was in line with earlier findings [31, 146, 157]. Here, we elaborated on the producer phenotyping concept and found that the interclass-bioactivation potential of gut microbiota varies from non-equol+non-8-PN+non-ENL producers to strong equol+8-PN+ENL producers. The prevalence of these phenotypes approximated the distribution of mutually independent events (*i.e.,* 15% non-producers, 8% equol, 7% 8-PN, 29% ENL, 4% equol+8-PN, 16% equol+ENL, 14% 8-PN+ENL, and 7% equol+8-PN+ENL producers). With some exceptions of enhanced metabolism, the formations of equol, 8-PN, and especially ENL were however generally lowered or even completely inhibited when their precursors were administered simultaneously.

Although these microbial metabolites are formed through different pathways, similar chemical conversions and rather closely related micro-organisms (demethylation [144, 158,

159] and reduction [159-162] are involved. Therefore, one of the explanations for the observed interferences upon cosupplementation may relate to competition for a common substrate, electron donor or cofactor. Hydrogen gas is formed in the colon by a variety of hydrolytic and saccharolytic bacteria to dispose reducing equivalents during fermentation [163], and has an important role in daidzein metabolism [160]. Competition for fermentative $H_2$ is well described [164] and equol-producing microbiota have been suggested to interact with the interspecies $H_2$ transfer [143]. Daidzein acts as an electron acceptor during equol formation and might divert $H_2$ away from ENL formation. On the other hand, a specific precursor or metabolite can directly or indirectly cause a selective stress or stimulus to specific phytoestrogen-metabolizing organisms. Similarly, the increased total phytochemical load potentially exceeds a minimal inhibitory concentration (MIC-value) or triggers an inducible pathway. Finally, a preferential metabolism (*e.g.,* demethylation of IX over SECO) may prevail, but more detailed experiments are required to unravel the underlying mechanisms.

Before testing whether overall health effects result from a combination of phytochemicals with multiple and perhaps additive or interfering activities, the first attention should go to the bioavailability and especially the microbial metabolism of isoflavones, prenylflavonoids, and lignans as part of a mixture, warranting the importance of this prospective study. Due to the study design, *i.e.*, the selective inclusion of multi-producers and multiple dosing of specific food supplements, literature reports of similar intervention trials are limited for the reference administrations, and lacking for the coadministrations. The urinary excretion of isoflavones and prenylflavonoids agreed with the results of similar dietary interventions with soy- and hop-derived food supplements administered separately to postmenopausal women classified as strong equol and 8-PN producers, respectively [146, 165]. Without differentiating between weak and strong ENL producers, the (entero)lignan concentrations measured in the urine samples were also in line with previous reports [166]. The impact of the timing of venipunctures, dosing regimen, and formulation on circulating phytoestrogen concentrations often hampers sound comparisons between dietary intervention trials. Nevertheless, we discuss the 'HOP'

interventions, since this and the study by Rad *et al.* [167] are the only reports on human serum prenylflavonoid concentrations. The latter found peak serum concentrations of approximately 400, 1800, and 5500 nmol/L of total 8-PN in healthy postmenopausal women after oral single doses of 50, 250, and 750 mg of 8-PN, respectively. Therefore, our results suggest cumulative effects caused by the multiple dosing protocol as described for isoflavones [149], and the administration of IX as a slow-release formation for 8-PN. The striking difference in relative 8-PN formation derived from the urine and serum samples (Figure 4.4 D *vs.* H) corresponds with the observations of Possemiers *et al.* [135] in human microbiota-associated rats, and likely reflects differences in pharmacokinetic behavior analogous to the consistently higher urinary and lower serum or plasma daidzein concentrations compared to genistein (Tables 4.1 and 4.2).

We translated the exposure to isoflavones, prenylflavonoids, lignans, and their microbial metabolites to an overall exposure to phytoestrogen-derived 17β-estradiol-equivalents towards estrogen receptor (ER) α ($E_2\alpha$) and ERβ ($E_2\beta$), based on the generally accepted dose-addition concept [166] and thereby assuming i) relative estrogenic potencies towards ERα of 1/5000, 1/100, 1/200, 1/10, and 1/10000, and towards ERβ of 1/100, 1/2, 1/35, 1/100, and 1/5000 for daidzein, genistein, equol, 8-PN, and ENL, respectively, [137-139], ii) binding to serum proteins of 81%, 55%, 50%, 50%, and 50% for daidzein, genistein, equol, 8-PN, and ENL, respectively, [169], and iii) an overall 98% attenuation due to glucuronidation and sulfatation [134, 167] (Table 4.3). Upon coadministration of soy, hop, and flax-based food supplements men (*n*= 10) were exposed to calculated 0.024-0.115 nmol/L $E_2\alpha$ and 0.180-1.336 nmol/L $E_2\beta$, whereas premenopausal women (*n*= 16) to 0.020-0.121 nmol/L $E_2\alpha$ and 0.261-1.603 nmol/L $E_2\beta$, and postmenopausal women (*n*= 9) to 0.042-0.137 nmol/L $E_2\alpha$ and 0.175-3.054 nmol/L $E_2\beta$. As a reference, the circulating 17β-estradiol ($E_2$) concentrations in men, pre-, and postmenopausal women are approximately 0.1 nmol/L, 0.15-1.00 nmol/L, and 0.015-0.050 nmol/L, respectively. The contribution of the isoflavones' estrogenicity towards ERβ (99.4%) was by far the most important one, whereas 8-PN was the most potent $E_2\alpha$ contributor (67.8%). Moreover, the exposure to $E_2\beta$ after intake of food supplements

containing only isoflavones exceeded significantly (Δ= 57.1%, *P*= 0.002) the superimposed activities of isoflavones, prenylflavonoids, and lignans in the mixture (Table 4.3). This observation and the lower bioactivation upon coadministration question the added value of food supplements combining various classes of phytoestrogens.

**Table 4.3:** **Calculated serum concentration of isoflavone-, prenylflavonoid-, and lignan-derived $E_2\alpha$ and $E_2\beta$-equivalents (mean±SEM) upon intake of soy-, hop-, and flax-based food supplements separately or simultaneously, and difference between these concentrations.**

| | Concentration, pmol/L | | Δ | |
|---|---|---|---|---|
| | **SOY/HOP/FLAX** | **MIX** | **%** | ***P*[#]** |
| **$E_2\alpha$** | | | | |
| **Isoflavones (*n*= 22)** | 34.2 ± 13.5 | 21.0 ± 1.9 | 47.0 | 0.001 |
| **Prenylflavonoids (*n*= 19)** | 60.4 ± 0.12 | 60.0 ± 10.4 | 63.9 | 0.107 |
| **Lignans (*n*= 21)** | 0.162 ± 0.014 | 0.150 ± 0.007 | 35.9 | 0.003 |
| **$E_2\beta$** | | | | |
| **Isoflavones (*n*= 22)** | 1399 ± 560 | 744 ± 85.5 | 52.6 | 0.003 |
| **Prenylflavonoids (*n*= 19)** | 6.04 ± 0.01 | 5.96 ± 1.04 | 63.9 | 0.107 |
| **Lignans (*n*= 21)** | 0.325 ± 0.028 | 0.299 ± 0.015 | 35.9 | 0.003 |

[#]Paired Student's *t*-test for all phytoestrogens under investigation.

In conclusion, this was the first intervention trial with a specific mixture of soy, hop, and flax-based food supplements, in which the microbial bioactivation of daidzein, IX, and SECO was taken into account to strengthen the estimates of maximal exposure to dietary phytoestrogen-derived $E_2$-equivalents. Further studies are needed to evaluate the impact of microbial bioactivation on the final health effects of mixtures of phytoestrogens and to extrapolate these results to the general population or to other phytoestrogenic mixtures *e.g.,* as would occur through dietary exposure. Nevertheless, this study revealed that the bioavailability of mixed phytoestrogens is strongly determined by the microbiome of each individual and personalized screenings prior the use of these combinations in supplements, are therefore highly recommended.

# CHAPTER 5

## Monoclonal antibodies against phytoestrogens

### *DEVELOPMENT AND PRODUCTION - VALIDATION OF ELISA*

This chapter is partially based on:

*Production of monoclonal antibodies against hop-derived (Humulus lupulus L.) prenylflavonoids and the development of immunoassays.*

Wyns, C., Derycke, L., Soenen, B., Bolca, S., Deforce, D., Bracke, M. and Heyerick, A.

Talanta, 2011. 85(1): p. 197-205.

# 1. INTRODUCTION AND OBJECTIVES

Generally the reported quantitative methods for the determination of phytoestrogens are based on reversed-phase liquid chromatography (RP-HPLC) combined with UV-detection or mass spectrometric detection (MS or tandem MS) [18, 101, 122, 170-172]. Those methods are rather expensive, and require experienced personnel and can be quite labor-intensive. Therefore, the access to immunoassays may offer a low-cost, high-throughput alternative to labs that do not have access or skills to do LC-MS(/MS). The first report of antibodies against phytoestrogens dates from 1969, when Bauminger *et al.* [173] reported the application of antibodies against genistein as a way of passive immunization and protection against interruptions of the reproductive system. This was soon followed by the development of several radioimmunoassays (RIAs) for phytoestrogens which are very sensitive, though, they mostly lack stability and cause waste and safety issues. Therefore, the conventional enzyme-linked immunoassays (EIAs) were introduced, as well as the immunoassays using fluorescent labeled antigen (FIA) and their sensitive variant, time-resolved immunoassays (TR-FIA). Schaefer *et al.* [40] have reported on the generation of polyclonal antibodies against 8-PN and the subsequent development of a radioimmunoassay for the quantitative determination of 8-PN in beer and urine. Lapcik *et al.* [174] also reported on a very specific immunoassay for coumestrol. Additionally, over the last few years, various papers have reported on immunoassays based on polyclonal antibodies for soy- or flax-derived phytoestrogens (daidzein, genistein and enterolactone) for the quantitation in biological samples [175-179]. Next to the application of monoclonal antibodies in quantitation methods, a wide range of other immunological techniques can be explored to elucidate metabolisation- and distribution profiles of phytoestrogens in biological matrices.

We preferred the technology of monoclonal antibodies, because they are known to show a highly specific binding to their haptens, to exert negligible cross-reactivities compared to polyclonal antisera and ensure the continuous production of a well-

characterized product. Literature search showed that monoclonal antibodies were developed only against daidzein, genistein by Kohen *et al.* [180, 181] and for the same compounds and equol by Talbot *et al*. [182]. In this study we aimed to develop monoclonal antibodies against a range of biologically relevant phytoestrogens: genistein, daidzein, equol, coumestrol, enterodiol, enterolactone, xanthohumol, isoxanthohumol and 8-prenylnaringenin. Although xanthohumol and isoxanthohumol are not estrogenic, the availability of monoclonal antibodies can be of great value during assays for the detection and distribution of phytoestrogens in several biological matrices. The generated monoclonal antibodies will be used to establish a competitive enzyme-linked immunosorbent assay (ELISA) using immunogen-coated microtitration plates and a peroxidase-labeled anti-mouse $IgG_1$ secondary antibody to measure concentrations in human biological fluids. Finally, the established ELISA will be validated for its use to measure concentrations of phytoestrogens in urine and serum samples.

# 2. EXPERIMENTAL

## *2.1 Chemicals and reagents*

Xanthohumol (XN), isoxanthohumol (IX), 8-prenylnaringenin (8-PN), desmethylxanthohumol (DMX), and 6-prenylnaringenin (6-PN) were available in our laboratory [52]. Equol (EQ) was purchased from Extrasynthèse (Genay, France). Daidzein (DAID), genistein (GEN) and naringenin were obtained from Acros Organics (Morris Plains, NJ, USA). Coumestrol (COUM), enterodiol (END), enterolactone (ENL) and β-estradiol (E2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), polyethylene glycol (PEG 1500), Tween-20, alkaline phosphatase-labeled anti-mouse IgG antibody, a Mouse Monoclonal Antibody Isotyping kit and SIGMA *FAST* para-nitrophenyl phosphate (*p*NPP) tablets were purchased from Sigma-Aldrich. All other chemicals and solvents for the synthetic work and supplements for the biological work were obtained from Acros Organics and were all of analytical reagent grade. The solvents for the chromatography were all of LC-MS quality and purchased from Biosolve (Valkenswaard, The Netherlands). Phosphate-buffered saline (PBS) solution was prepared as 0.137 M NaCl and 2.7 mM KCl in 0.01 M phosphate buffer, pH 7.4. Complete serum-free Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) was supplemented with L-glutamine (0.05%), penicillin (100 units/mL), and streptomycin (100 $\mu$g/mL). The murine NS0 cell line, a non-secreting plasmacytoma cell line (myeloma) from Balb/c mice was kindly provided by the Laboratory for Cellular Immunology (P. De Baetselier, Vrije Universiteit Brussel, Belgium). For the cultivation of the NS0 cell line complete DMEM was supplemented with heat-inactivated FBS (10%, Invitrogen) and thioguanine (10 µM). HAT medium was used to select and culture hybridomas and consisted of complete DMEM with heat-inactivated FBS (20%), hypoxanthine (120 µM), thymidine (19 µM) and aminopterin (0.6 µM). Dialysis of the antigen conjugates and cell media was performed with Slide-A-Lyzer cassettes (0.5-3.0 mL; MWCO 7000 Da and 12.0-30.0 mL; MWCO 10 kDa) from Pierce (Rockford, IL, USA). For the production of monoclonal antibodies large scale Integra Biosciences CELLine bioreactors (type CL350) were used and were purchased from Integra Biosciences (Chur, Switzerland).

For the purification of the monoclonal antibodies, HiTrap protein G columns from GE Healthcare (Piscataway, NJ, USA) were used. The ultracentrifugation was performed with Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA, USA). For the protein gel electrophoresis TEMED, sodium dodecyl sulfate-polyacrylamide (SDS) and β-mercapto-ethanol were purchased from Bio-Rad Laboratories (Richmond, CA, USA) and all other used products were from Acros. The acetate buffer (0.1 M; pH 5.0) was made from sodium acetate hydrate obtained from UCB (Leuven, Belgium). The enzymatic deconjugation solution (33 mg/mL) was prepared in acetate buffer with sulfatase from *Helix pomatia* (type H-1 with sulfatase activity: 15.3 units/mg and β-glucuronidase activity: 300 units/mg) from Sigma-Aldrich. Peroxidase-labeled anti-mouse $IgG_1$ antibody, unlabeled mouse IgG antibody and ABTS stop solution were obtained from SouthernBiotech (Birmingham, Alabama, USA). ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) tablets and peroxidase substrate buffer were purchased from Pierce. The ELISA technique was carried out on microtiter plates (Nunc Maxisorp, Nunc, Roskilde, Denmark) with 96 wells.

### *2.2 Instrumentation*

Semi-preparative chromatography was performed with a Varian Omnisphere $C_{18}$ column (250 mm x 21,4 mm, 10 µm) from Varian (Palo Alto, CA, USA) using a Gilson 322 pump (Gilson, Middleton, WI, USA) with a Gilson UV-VIS 156 detector and a Gilson 206 fraction collector. The identity of the haptens was confirmed by interpretation of proton magnetic resonance spectra ($^1$H NMR, 300 MHz) and carbon magnetic resonance spectra ($^{13}$C NMR, 75 MHz) data obtained with a Varian Mercury-VX 300 MHz spectrometer. Thin-layer chromatography (TLC) was performed using silica-coated aluminium plates with fluorescent indicator (Alugram SIL G/UV 254 plates, 0.2 mm; Macherey-Nagel & Co., Düren, Germany) and TLC separations were examined under UV light at 254 nm and 350 nm and revealed by a sulfuric acid-anisaldehyde spray. The hapten/carrier protein ratio of the antigen conjugates was estimated by UV-spectrometry at characteristic wavelengths using a Shimadzu UV-1600PC spectrophotometer (Shimadzu, Kyoto, Japan) in scan mode in a 1 cm quartz cuvette and using UV Probe 2.20 software. Purification of the produced monoclonal antibodies was performed on an AKTA-FPLC apparatus (GE Healthcare). Microtiter plates from the primary

screening assays were read with a Molecular Devices (Wokingham, Berkshire, UK) microplate reader and the absorbances of the competitive immunoassays were determined with a Beckman Coulter Paradigm Detection Platform (Fullertan, CA, USA). The HPLC-MS system was an Agilent 1200 (Agilent Technologies, Waldbronn, Germany) equipped with vacuum degasser (G1379B), binary pump (GG1312A), autosampler (G1329A), column thermostat (GG1316A) and diode array detector (G1315B). The system was coupled online to the mass selective MSD detector (G1956B), SL version, controlled by Chemstation software (Rev. B 02.01) and the MSD was tuned with Agilent APPI/APCI tuning mix. Statistical analyses during validation were performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

### *2.3 Synthesis of phytoestrogen derivatives and immunogen synthesis*

The molecular weight of phytoestrogens is too low (< 1000 Da) to evoke immune responses, and consequently immunization had to be conducted with conjugates, *i.e.* haptens (phytoestrogen + linker) covalently linked to an immunogen protein, BSA. The resulting conjugate is immunogenic and antibodies will be produced against the low-molecular non-protein part. The linker is a short spacer with a carboxylic acid group, and is held as small as possible to restrict flexibility and to result in an immune response as high as possible. The carboxylic acid group serves for the chemical coupling to an ε-amino group of a lysine-residu of BSA. To ensure the specificity of the antibodies, the linker was positioned in favor of a minimal structural loss of the antigen molecule. Therefore the haptenisation was conducted at the biological least important position, which is the 4'-OH group (except for coumestrol, which was linked to BSA with the 3-OH group).

The first step in the synthesis was the formation of ethoxycarbonylmethylderivatives of the targeted phytoestrogens. The *O*-carboxymethylderivatives 4'-*O*-carboxymethyl-genistein, -daidzein and -xanthohumol were prepared by selective alkylation of the phenolic 4'-hydroxy group using dry potassium-tert-butoxide and ethyl bromoacetate in dry DMF (HMPA for XN) according to Al-Maharik *et al.* with minor modifications [183]. For coumestrol, 3-*O*-carboxymethylcoumestrol was prepared using ethyl chloroacetate in

potassium carbonate. After alkylation the ethylester was hydrolyzed under acidic conditions to the desired acid in good yield. The 4'-*O*-carboxymethyl-derivative of isoxanthohumol was obtained by cyclization of the xanthohumol-derivative under alkaline conditions. For the preparation of the 4'-*O*-carboxymethyl-derivative of 8-prenylnaringenin synthesis was started from naringenin. After protection of the 7-OH-group and subsequent linking with the spacer, naringenin was prenylated at the 5-OH-group [184]. Through a Claisen rearrangement the 8-prenylnaringenin-derivative was obtained.

Secondly, after the coupling of the phytoestrogen to the spacer, the resulting product had to be conjugated to BSA. For activation of the haptens the dicyclohexylcarbodiimide (DCC)/N-hydroxysuccinimide (NHS) coupling method of Hosoda *et al.* [185] was adapated. Briefly, a solution of the haptens was put for 2 to 5 hours at room temperature with DCC and NHS in dry DMF. The mixtures were then centrifuged to deposit the crystals of dicyclohexylurea. The supernatants containing the respective NHS-esters were conjugated with BSA in a reversed micellar system [186] at a molar ratio (between the activated compound and BSA) of approximately 70:1 which is sufficient for a high degree of haptenisation, since approximately 60 amino groups can be used for modification. As there is a positive correlation between the number of haptens incorporated and the buffer/octane ratio, a large amount of buffer was used (6/1 buffer/octane). Upon clearance of the mixture, the activated haptens in DMF solution were added immediately under vigorous shaking. After a slight transient turbidity was observed the reaction mixture was allowed to react 24 to 36 h at room temperature. Total disappearance of the activated hapten was checked by TLC (4/1 trichloromethane/methanol). Hapten-BSA conjugates (Figure 5.1 depicts the conjugates for XN, IX and 8-PN) were isolated from the mixture by precipitation with three volumes cold acetone at 0 °C followed by centrifugation. The supernatant was removed, the sediment was washed against $PBS^{D-}$ and dialyzed overnight at 4 °C under continuous stirring against $PBS^{D-}$ using Slide-a-Lyzer cassettes. The remaining solution was filtered through a 0.2 µm filter and lyophilized. The hapten/carrier protein ratio of the conjugates was determined by spectrophotometric analysis. Aliquots of the conjugates were stored at -20 °C until use.

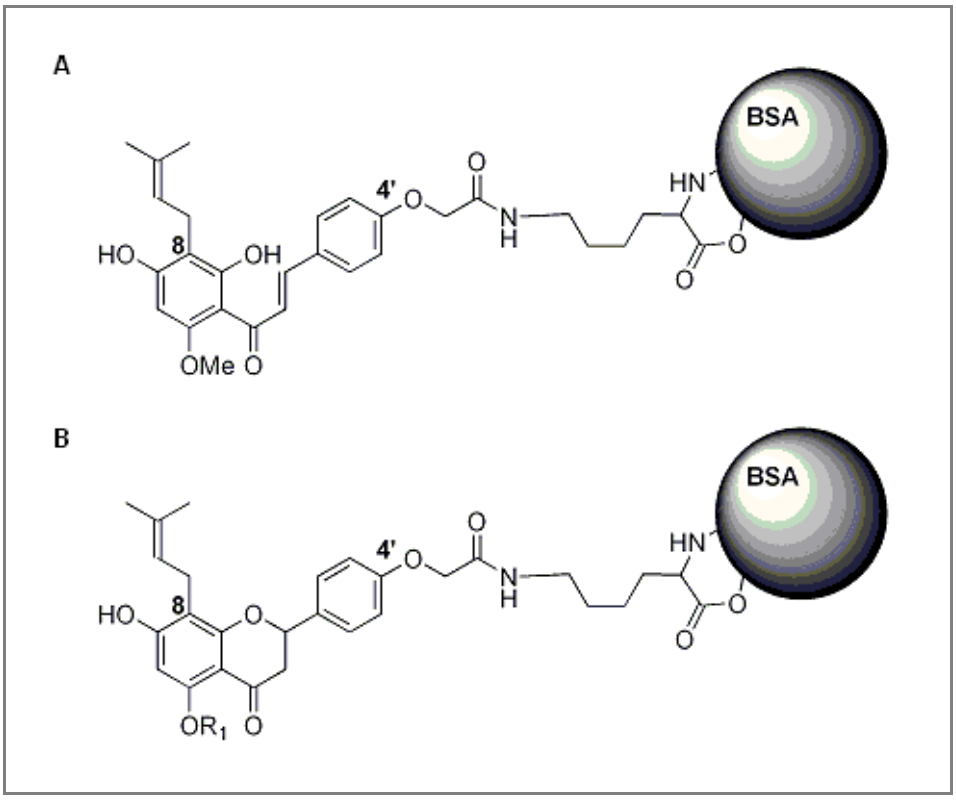



**Figure 5.1:** **Structures of three immunogens: (A) 4'-*O*-carboxymethyl-xanthohumol-BSA and (B) 4'-*O*-carboxymethylisoxanthohumol-BSA ($R_1$= Me); 4'-*O*-carboxymethyl-8-prenylnaringenin-BSA ($R_1$= H).**

## *2.4 Immunization*

Three to four BALB/c female mice (12 to 16 weeks old) (Charles River Laboratories, Wilmington, DE, USA) were immunized subcutaneously (100 µL) with the respective hapten-BSA conjugate (100 µg). Two and four weeks after the initial injection, booster injections were given intraperitoneally with an equal amount of immunogen. Three days after the last booster injection the mice were sacrificed for lymphocyte/myeloma cell fusion. Afterwards their spleens were isolated and transferred to 10 mL DMEM.

### 2.5 Cell fusion

The procedure described by Brown and Ling [187] was used as basis for the cell fusion. Murine NS0 myeloma cells were cultured in complete DMEM supplemented with 10% FBS. Mice spleens were perfused with 20 mL serum-free DMEM, and the resulting lymphocyte suspension was mixed with the myeloma cells at a ratio of 10:1 and centrifuged. A few drops of alkaline DMEM were added to the cell pellet and after rolling of the tube 700 µL of a 50% PEG solution (w/v, in 1 mM NaOH) at 37 °C was added. Next, the procedure was completed with alternating of rolling steps and the addition of (in total) 15 mL serum-free DMEM. Finally, after completion of the fusion procedure, the cells were spun down and suspended in 20 mL of HAT selection medium. The resulting cell suspension was distributed (100 µL $well^{-1}$) in 96-well tissue culture plates and incubated in a 10% $CO_2$ incubator at 37 °C. The next day one drop of HAT medium was added to the wells and every two days culture medium was replaced.

### 2.6 Hybridoma selection and cloning

Two weeks after the cell fusion, culture supernatants from all fusion wells were screened for the presence of anti-hapten antibodies. Initial screening and selection was performed using two replicate non-competitive enzyme-linked immunosorbent assays (ELISA) with the conjugates of the phytoestrogens as the coating antigens. Next, antibodies from the selected hybridomas were tested in a competitive indirect ELISA to determine whether they recognize pure XN/IX/8-PN and the coating antigen on a competitive basis. The monoclonality of the produced antibodies was ensured via limiting dilution (two rounds of subcloning) of the selected hybridomas [187]. Several aliquots of the first hybridomas and their clones were cryopreserved in liquid nitrogen (in 10% FBS in DMSO) at several stages during the development.

## 2.7 Production and characterization

The clones of interest were cultured for a period of 3 up to 5 months *in vitro* in Integra CELLine bio-reactors. After dialysis against PBS using Pierce Slide-A-Lyzer cassettes, monoclonal antibodies (mAbs) were purified from the collected culture media with fast protein liquid chromatography (FPLC) by their affinity for HiTrap Protein G columns and, subsequently, ultrafiltered against PBS. The protein concentration of the final antibody solutions was determined using a Bio-Rad protein concentration determination assay. Then, using the Sigma mouse monoclonal antibody isotyping kit, the isotype class was determined. Finally, the purity of the antibody solutions was confirmed by 12% SDS gel electrophoresis, followed by Coomassie blue staining [188]. The mAb stocks were stored at a 1 mg/mL concentration in PBS at -20 °C until further use.

## 2.8 Indirect non-competitive screening ELISA

The selection of the hybridoma's was carried out using an antigen-coated ELISA, with supernatants from the hybridoma cultures. Briefly, microtiterplates were coated overnight at 4 °C with 100 µL of a 10 µg/mL solution of phytoestrogen conjugates. After washing four times with PBS containing 0.05% (v/v) Tween-20 (PBS-Tw), the wells were blocked for 1 h at 37 °C with 1% BSA in PBS. Next, after washing, 100 µL of supernatant was added and incubated during 2 h at 37 °C. After washing, 100 µL of the alkaline phosphatase-labeled rabbit anti-mouse IgG antibody at a 1:3000 dilution was added and incubated for 1 h at 37 °C. Finally, after a final washing step, 100 µL of the *p*NPP substrate solution was added at room temperature and after 30 minutes the absorbance was measured at 405 nm.

## 2.9 Indirect competitive ELISA

After two screenings with the non-competitive ELISA, the affinity of the antibodies from the selected hybridomas for XN, IX or 8-PN was tested in an indirect, but competitive ELISA. Briefly, the same procedure as in section 2.8 was applied, except for the extra addition of

pure XN, IX or 8-PN (10 $\mu$L of a 1 mg/mL solution in methanol) together with the culture supernatants to the wells. Whenever the binding of the antibodies to the coating antigen was inhibited by their affinity for XN, IX or 8-PN, thereby decreasing the absorbance, the respective hybridomas were included for further experiments. Next, the different steps and parameters of this competitive ELISA were thoroughly optimized, using the purified monoclonal antibodies. In summary, checkerboard titrations were completed to establish the optimal working dilutions of the coating antigen, blocking buffer, primary and secondary antibody, and the type of secondary antibody and enzymatic substrate, incubation temperatures and time periods were optimized. Furthermore, the influence of organic solvent (*i.e.*, methanol) for dissolvation of the prenylated flavonoid standards, on assay performance was examined.

The optimized procedure for the competitive ELISA was as follows: wells were coated overnight (at least 12 h) at 4 °C with 200 µL of a 0.5 µg/mL solution of the respective antigen and after washing with PBS-Tw blocked with 250 µL of 3% BSA in PBS during 1 h at 37 °C. Then, per well 100 µL of serial dilutions of the competitor, *i.e.* XN in 50% methanol in PBS or IX or 8-PN (in 10% methanol in PBS) and an equal volume of the specific antibodies (5 ng/mL in PBS with 0.1 % BSA) were added and incubated during 2 h at 37 °C under slow agitation. In negative control (NC) wells, the primary antibody was replaced by an antibody of irrelevant specificity (unlabeled mouse IgG; 1:5000 dilution in 0.1% BSA in PBS). The plate was washed with PBS-Tw and 200 µL of peroxidase-labeled anti-mouse $IgG_1$ antibody was added to each well. The plate was left at 37 °C for one hour. After washing the plate four times with PBS-Tw, 200 µL ABTS substrate solution in peroxide substrate buffer was added and reaction took place in the dark at room temperature and was then stopped after 30 min by the addition of stop solution. Absorbance was measured at 405 nm and calibration curves were constructed by plotting $[(B_i\text{-NC})/(B_0\text{-NC})]$ ($B_i$= absorbance of wells with standard/biological sample; $B_0$= absorbance from maximum binding of the antibody to the antigen without competition) against log(competitor concentration) and fitted to a four-parameter logistic equation using Sigmaplot version 11.0 (Systat software, Chicago, IL, USA). For each point of the calibration curve a minimum of five replicates was used and absorbances were the mean

value of duplicate readings. From the equation of the calibration curves, $IC_{50}$ values (concentration at which the binding of the antibody to the coating antigen is inhibited with 50%) were determined and subsequently used to express the sensitivity and the detection range of the ELISA.

### *2.10 Determination of cross-reactivity*

A panel of phytoestrogens (daidzein, genistein, enterodiol, enterolactone, equol, coumestrol), 17 β-estradiol and structurally related compounds (desmethylxanthohumol, 6-prenylnaringenin) were tested for cross-reactivity by determining their respective $IC_{50}$ values in the competitive assay as described above. Cross-reactivity values were calculated as the ratio of the $IC_{50}$ of the XN, IX or 8-PN standard to the $IC_{50}$ of the test compounds and expressed as a percentage.

### *2.11 Preparation of samples for validation and extraction of urine and serum*

For the validation of the competitive ELISA, spiked urine and serum samples, as well as some urine samples from the dietary intervention study described in Chapter 4 [130], were prepared. Briefly, for the hydrolysis of glucuronide and sulfate conjugates, one volume of (spiked) urine or serum was mixed with one volume of sodium acetate buffer and incubated with a β-glucuronidase/sulfatase solution overnight at 37 °C. Next, the samples were extracted using five volumes of diethyl ether under vigorous vortex mixing during 30 seconds. This extraction was repeated three times to ensure complete recovery of the compounds. Finally, the collected solvent layers were evaporated to dryness under nitrogen and the residue was dissolved in assay buffer (50% methanol in PBS for XN and 10% methanol in PBS for IX/8-PN).

# 3. RESULTS AND DISCUSSION

## *3.1 Monoclonal antibody production*

Conjugates with BSA were generated for daidzein, genistein, coumestrol, xanthohumol, isoxanthohumol and 8-prenylnaringenin. The hybridomas for the respective phytoestrogens were constructed. After the initial screening phase only the hybridomas against coumestrol, xanthohumol, isoxanthohumol and 8-prenylnaringenin were retained. Talbot *et al.* [182] described in 2008 the development and application of monoclonal antibodies against daidzein and genistein, and therefore, we did not give priority to this issue. The antibodies generated from the remained hybridomas were tested according to the competitive ELISA as described in section 2.9 of this chapter. For XN, IX and 8-PN the affinity of the antibodies for the pure phytoestrogen could be guaranteed. The development of antibodies against coumestrol was stopped, due to lack of conjugate stock. Figure 5.2 depicts a colony from 1 cell of the XN-hybridoma, at the 2$^{nd}$ cycle of cloning. Monoclonality of the antibodies generated from the three retained hybridoma cell lines (XN, IX and 8-PN) was tested by the procedure written in section 2.6 of this chapter.

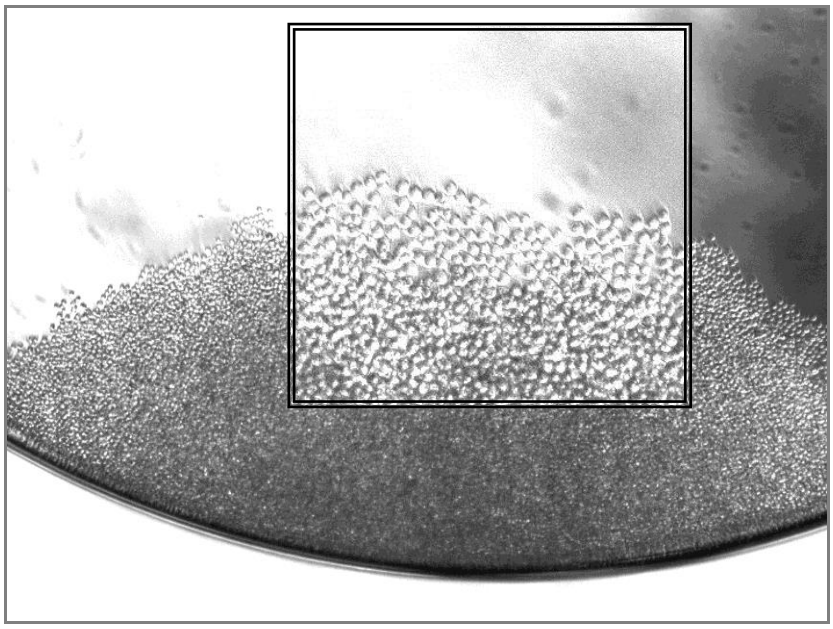


**Figure 5.2:** Hybridoma colony (in a well of a 96-well plate) (5x magnification).

Large quantities of anti-XN, anti-IX and anti-8-PN were produced and then purified, and consequently 59 mg, 195 mg and 52 mg of total protein was generated, respectively. Figure 5.3 depicts the result of the protein gel electrophoresis of the monoclonal antibodies, which are all of the $IgG_1$ isotype, and shows their purity and lack of contamination with BSA (= 65 kDa).

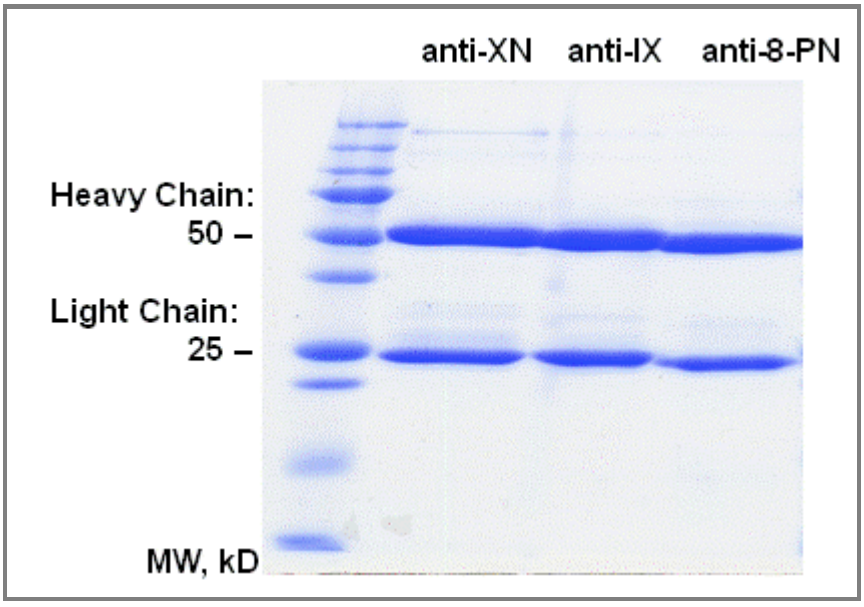



**Figure 5.3:** Gel demonstrating purity of developed monoclonal antibodies (anti-XN, -IX and -8-PN).

### *3.2 Immunoassay optimization*

The concentrations of XN, IX and 8-PN in biological samples, upon ingestion of a hop-based dietary supplement, most often only reach to lower ppb-levels. Therefore, the analytical sensitivity of the competitive ELISA, which is represented by its $IC_{50}$ value, has to be as low as possible. Consequently, the amount of primary antibody and conjugate, as well as the concentration and type of blocking buffer (1% to 3% BSA in PBS or 0.5% milk powder in PBS), the amount of secondary antibody (1:2000 to 1:8000), type of substrate (ABTS, TMB or OPD) and the duration (0.5 to 2 h) and incubation temperature (room temperature or 37 °C) of each step were optimized in order to enhance the analytical sensitivity. The standard curve characteristics were optimal at conjugate and antibody concentrations of 0.5 µg/mL and 5 ng/mL, respectively, for XN, IX and 8-PN and the other parameters were defined as described in section 2.9 of this chapter.

Furthermore, the effects of different concentrations of methanol on the performance of the ELISA were studied. Given the relatively apolar nature of XN, IX and 8-PN, a certain amount of organic solvent is necessary to ensure full solubilization in PBS. Although DMSO is often used for this purpose, we opted for methanol, as it is generally the best tolerated solvent in ELISA. Methanol dissolves XN, IX and 8-PN very well and we did not notice any difference in solubility with the former. Consequently, a compromise had to be found between solubility and influence of methanol on assay performance. For that, the maximum signal or the maximum binding of the antibody was observed with increasing amounts of methanol added to the assay buffer (Table 5.1; amount of methanol expressed as percentage of the well). For the anti-IX and anti-8-PN antibodies the binding was attenuated starting from 12.5% methanol in assay buffer. The binding of anti-XN, on the other hand, benefits from increasing amounts of methanol, and only starts to decrease with 50% methanol in the well. The solubility of XN, IX and 8-PN in PBS with added amounts of methanol in the assay buffer, respectively 50% for XN and 10% for IX and 8-PN, was examined by triplicate analysis with HPLC-UV (quantification at 370 nm for XN and 295 nm for IX and 8-PN) [189]. The signal intensity of XN, IX and 8-PN (1.25 µg/mL) in increasing

methanol concentrations versus the same concentration in 100% methanol (or 100% dissolvation) was investigated. Analysis results showed that the solubility of XN increased from 76.10% in 1% methanol in PBS to 98.70% in 50% methanol in PBS. For IX and 8-PN (both at a concentration of 1.25 µg/mL), the solubility increased from 84.40% and 83.30% in 1% methanol in PBS to 97.03% and 97.97% in 10% methanol, respectively. Additionally, calibration standards were prepared just before the beginning of each assay, to secure full solubilization and to minimize degradation of the analytes in PBS.

**Table 5.1:** **Influence of methanol (expressed as percentage in the well) on the binding capacity of the primary antibodies (0% methanol as reference)(*n*=6).**

| Relative binding capacity (%) | | | |
|---|---|---|---|
| MeOH (%) | *anti*-XN | *anti*-IX | *anti*-8-PN |
| **0** | 100 | 100 | 100 |
| **0.25** | 111 | 92 | 98 |
| **0.5** | 106 | 93 | 107 |
| **2.5** | 123 | 92 | 106 |
| **5** | 139 | 97 | 106 |
| **12.5** | 155 | 90 | 94 |
| **25** | 155 | 85 | 81 |
| **50** | 75 | 81 | 77 |

Figure 5.4 depicts the color development in a standard 96-well plate in a completed competitive ELISA for XN.

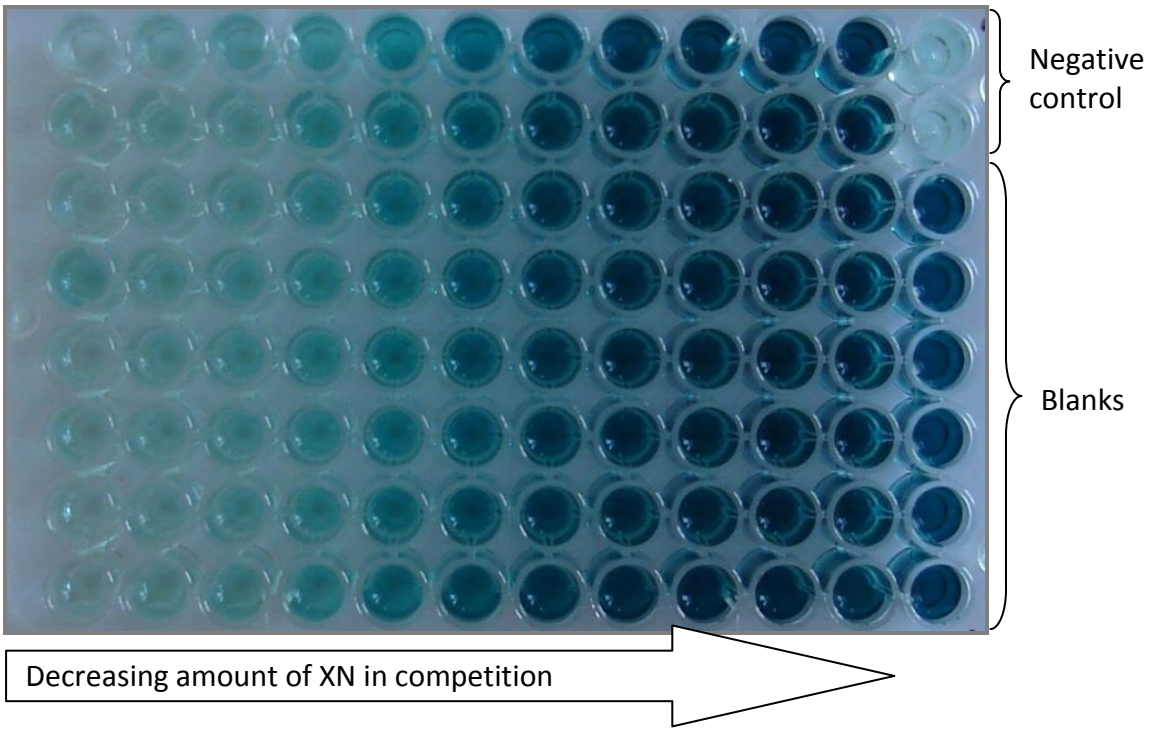
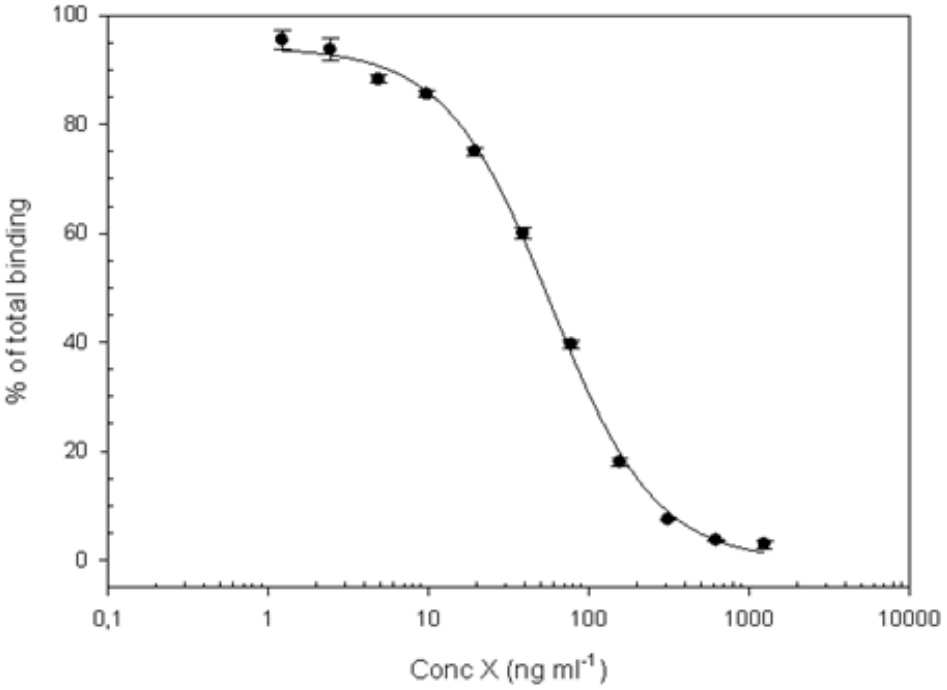


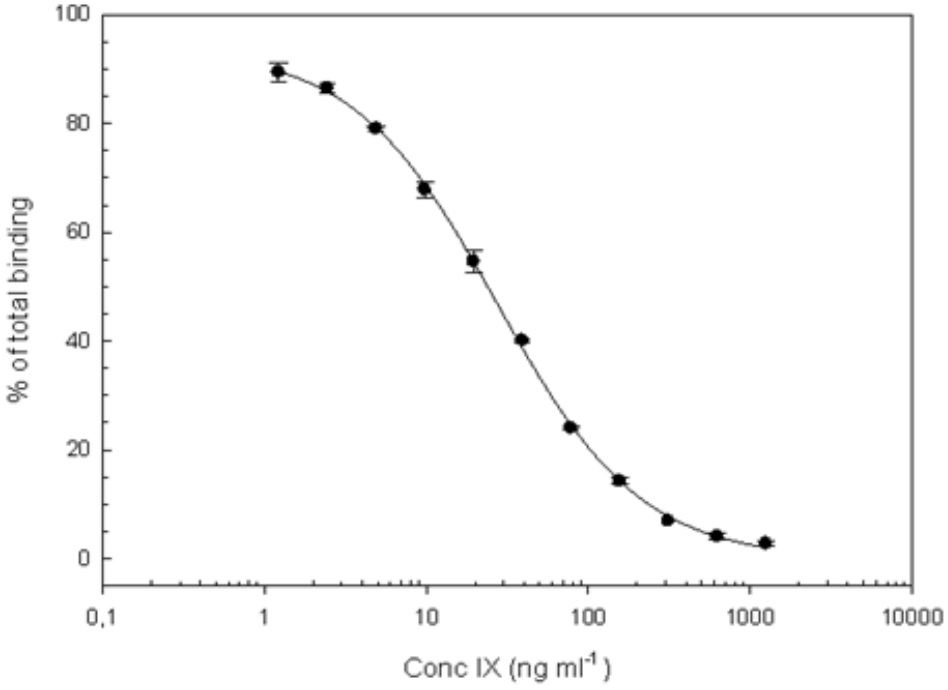

**Figure 5.4:** **View of 96-well plate with standard calibration curve for XN (2500 ng/mL – 1,22 ng/mL in competition in ELISA).**

Standard curves (mean ± SD) for XN, IX and 8-PN from 15 assays, measured on three different days under optimized conditions, are shown in Figure 5.5 (A, B and C). The $IC_{50}$ values were 62.91 ng/mL for anti-XN, 37.15 ng/mL for anti-IX and for anti-8-PN and their respective limits of detection (10% inhibition) were 14.45 ng/mL, 5.62 ng/mL and 4.37 ng/mL. Values for the LOQ (limit of quantification) were calculated as 10 x SD of the blank and were 41.01 ng/mL, 13.90 ng/mL and 17.10 ng/mL for XN, IX and 8-PN, respectively.

A)


100
80
60
40
20
0
% of total binding
0,1
1
10
100
1000
10000
Conc X (ng ml-1)

B)


100
80
60
40
20
0
% of total binding
0,1
1
10
100
1000
10000
Conc IX (ng ml-1)

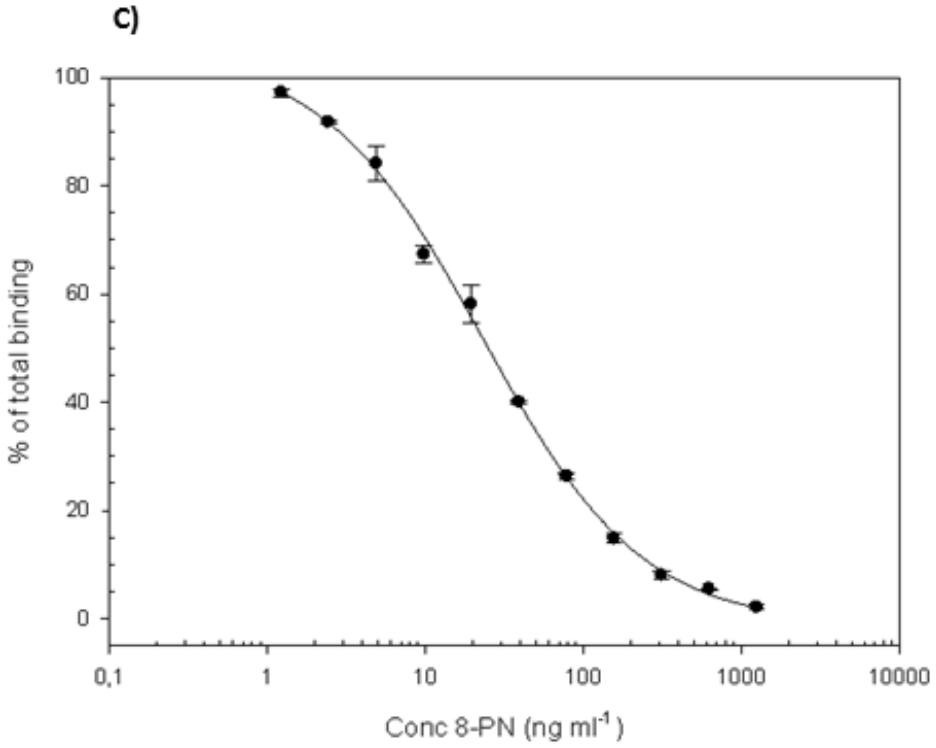



**Figure 5.5:** **Standard curves for xanthohumol (A), isoxanthohumol (B) and 8-prenylnaringenin (C) obtained with the optimized ELISA, means based on 15 replicate calibration curves (*n*=15) on three different days (± standard deviation).**

### *3.3 Specificity of the monoclonal antibodies*

The cross-reactivities of the three monoclonal antibodies against a panel of other phytoestrogens, chemically related compounds and 17β-estradiol (E2) are shown in Table 5.2. Generally, all three antibodies revealed excellent specificity for their antigens. For anti-8-PN the negligible cross-reactivity (<0.03%) presented by DMX and 6-PN is probably due to the impurity of the respective standards or in situ isomerization (for DMX). On the other hand, anti-XN showed some, negligible cross-reactivity (<0.05%) with IX and 8-PN.

**Table 5.2:** Cross-reactivity of three monoclonal antibodies against structurally related compounds.

| Cross-reactivity (%) | | | |
|---|---|---|---|
| competitor | *anti*-XN | *anti*-IX | *anti*-8-PN |
| **GEN** | < 0.01 | < 0.01 | < 0.01 |
| **DAID** | < 0.01 | < 0.01 | < 0.01 |
| **END** | < 0.01 | < 0.01 | < 0.01 |
| **ENL** | < 0.01 | < 0.01 | < 0.01 |
| **EQ** | < 0.01 | < 0.01 | < 0.01 |
| **COUM** | < 0.01 | < 0.01 | < 0.01 |
| **DMX** | < 0.01 | < 0.01 | < 0.03 |
| **6-PN** | < 0.01 | < 0.01 | < 0.03 |
| **E2** | < 0.01 | < 0.01 | < 0.01 |
| **XN** | 100 | < 0.01 | < 0.01 |
| **IX** | < 0.05 | 100 | < 0.01 |
| **8-PN** | < 0.05 | < 0.01 | 100 |

### *3.4 Analytical recovery*

The recovery after solvent extraction with diethyl ether was assessed by spiking a known amount of XN, IX or 8-PN in blank urine and serum before extraction and after extraction. By comparing the measured concentrations (from triplicate analyses) at low (= $QC_1$; 50 ng/mL), medium (= $QC_2$; 250 ng/mL) and high (= $QC_3$; 750 ng/mL) QC level from the competitive ELISA, the extraction recovery and the possible influence of matrix components are assessed. In Table 5.3 the mean recoveries (with coefficients of variation) are presented for XN, IX and 8-PN, for three concentration levels in urine and serum. In urine the recoveries for XN ranged from 96.3 % to 110.4%, for IX from 89.7% to 96.9% and for 8-PN from 89.1% to 97.1% and in serum the recoveries ranged from 85.0% to 95.3% for XN, 97.0% to 100.6% for IX and 85.7% to 95.3% for 8-PN. It can be concluded, from these recovery experiments, that the suggested extraction method offers a simple sample preparation with satisfying recoveries of all three analytes at physiological relevant levels.

**Table 5.3:** **Recovery of XN, IX and 8-PN in spiked urine and serum samples after solvent extraction at three QC levels.**

| | Recovery (CV) (%) | XN | IX | 8-PN |
|---|---|---|---|---|
| URINE | low | 110.38 (0.76) | 89.68 (1.59) | 89.05 (3.65) |
| | medium | 103.87 (0.22) | 96.44 (3.28) | 95.61 (0.95) |
| | high | 96.26 (3.67) | 96.88 (0.25) | 97.13 (3.52) |
| SERUM | low | 95.34 (8.43) | 97.65 (2.45) | 85.71 (7.07) |
| | medium | 84.98 (4.44) | 96.99 (4.00) | 90.92 (1.07) |
| | high | 88.05 (2.33) | 100.58 (3.76) | 95.28 (1.24) |

### *3.5 Matrix effects*

The influence of certain matrix components in urine and serum was assessed, because they may interfere with the affinity of the antibody for its antigen. Our main goal was to develop a short and easy ELISA and therefore the possibility of measurement of unprocessed urine or serum samples was investigated. Blank urine and serum were serially diluted in PBS and a constant amount of antibody was added. Then, the changes in maximal absorbance were monitored (Table 5.4). For anti-XN, in urine a minimum dilution factor of 10 was required to obtain at least 80% of the maximum signal and in serum at least a 100-fold dilution is required. For IX and 8-PN in serum, the effect of matrix components on the maximum absorbance was so large that at least 50-fold dilutions are recommended. In urine, the influence of the matrix on the binding capacity of anti-IX and anti-8-PN was smaller. As a result, we concluded that the influence of matrix components on the performance of the ELISA was substantial and variable.

**Table 5.4:** **Influence of urine and serum on the maximum binding of the antibodies (without preliminary extraction).**

| % matrix (in PBS) | | $A_{max}$ (%) | | |
|---|---|---|---|---|
| | | anti-XN | anti-IX | anti-8-PN |
| URINE | 100 | 35.71 | 60.24 | 40.86 |
| | 50 | 48.20 | 76.48 | 52.41 |
| | 20 | 63.49 | 85.20 | 70.28 |
| | 10 | 81.02 | 99.60 | 88.35 |
| | 4 | 82.79 | 90.24 | 90.26 |
| | 2 | 94.32 | 89.76 | 96.29 |
| | 1 | 91.43 | 97.52 | 96.49 |

| % matrix (in PBS) | | $A_{max}$ (%) | | |
|---|---|---|---|---|
| | | anti-XN | anti-IX | anti-8-PN |
| SERUM | 100 | 41.63 | 56.32 | 60.69 |
| | 50 | 46.39 | 57.33 | 62.96 |
| | 20 | 46.75 | 65.94 | 70.25 |
| | 10 | 54.99 | 74.55 | 71.43 |
| | 4 | 62.21 | 77.27 | 77.34 |
| | 2 | 70.81 | 84.33 | 81.97 |
| | 1 | 78.13 | 89.60 | 94.68 |

Therefore, for the rest of the validation an extraction of urine and serum samples prior to the ELISA was recommended. The matrix effect was then also assessed at three QC levels, by comparison of spiked samples after extraction (100% recovery) and the spikes in assay buffer. As shown in Table 5.5, the extraction step with diethyl ether substantially improved the assay performance. In urine, matrix effects gave an average underestimation of the final concentrations of 9.6% to 13.2% for XN, whereas for IX and for 8-PN matrix effects resulted in an underestimation of 0.8% to an overestimation of 9.6%. As expected, in serum, the underestimation of the final concentrations was slightly higher than in urine, and ranged for XN from 6.1% to 18.2%, for IX from 16.0% to 21.9% and for 8-PN from 13.4% to 19.8%.

**Table 5.5:** **Matrix effects of extracted urine and serum samples on assay performance at three QC levels.**

| | ME (%) | XN | IX | 8-PN |
|---|---|---|---|---|
| URINE | low | 86.91 | 86.91 | 99.23 |
| | medium | 86.82 | 86.82 | 104.73 |
| | high | 90.45 | 90.45 | 109.61 |
| SERUM | low | 93.92 | 78.10 | 86.58 |
| | medium | 84.49 | 84.05 | 81.43 |
| | high | 81.84 | 82.95 | 80.24 |

### *3.6 Precision and accuracy*

Three quality control samples of XN, IX and 8-PN in urine and serum were analyzed twice in three replicates in the same competitive assay on the same day, as well as in identical assays that were performed on three consecutive days and the inter- and intra-assay variations were calculated as %CV (Table 5.6). For urine and serum, the inter-assay variation was overall lower than 10%, except at the $QC_1$ level of XN and 8-PN in serum, where the variation was 14.7% and 12.3%, respectively. The relative standard deviations were below 5% for the intra-assay variation, both in urine and serum and for all three QC levels. Certain factors, like evaporation of the solvents at the edge of the wells, non-homogeneity of the temperature or slight changes in the preparation of buffers and solutions, can cause these variations. Therefore, the use of daily calibration curves is highly recommended. Furthermore, accuracy was assessed as the percentage relative error and never exceeded 15%, both in urine and in serum (Table 5.7). In conclusion, the obtained results for the accuracy and precision assessment met the acceptance criteria in analytical

validation guidelines [189], with emphasis on important issues described by Findlay and co-workers [190].

**Table 5.6:** Inter- and intra-assay variation (%CV) in urine and serum at three QC levels (*n*=3).

| | | URINE | | | SERUM | | |
|---|---|---|---|---|---|---|---|
| | QC level | XN | IX | 8-PN | XN | IX | 8-PN |
| INTER-ASSAY | low | 1.8 | 5.2 | 9.5 | 14.7 | 4.7 | 12.3 |
| | medium | 3.0 | 8.8 | 7.0 | 7.6 | 7.0 | 2.7 |
| | high | 2.2 | 4.7 | 5.0 | 4.4 | 6.7 | 3.4 |
| INTRA-ASSAY | low | 1.7 | 3.0 | 0.7 | 1.9 | 1.9 | 2.9 |
| | medium | 2.1 | 1.3 | 0.4 | 0.5 | 1.3 | 1.9 |
| | high | 1.9 | 2.9 | 1.1 | 1.7 | 1.5 | 2.5 |

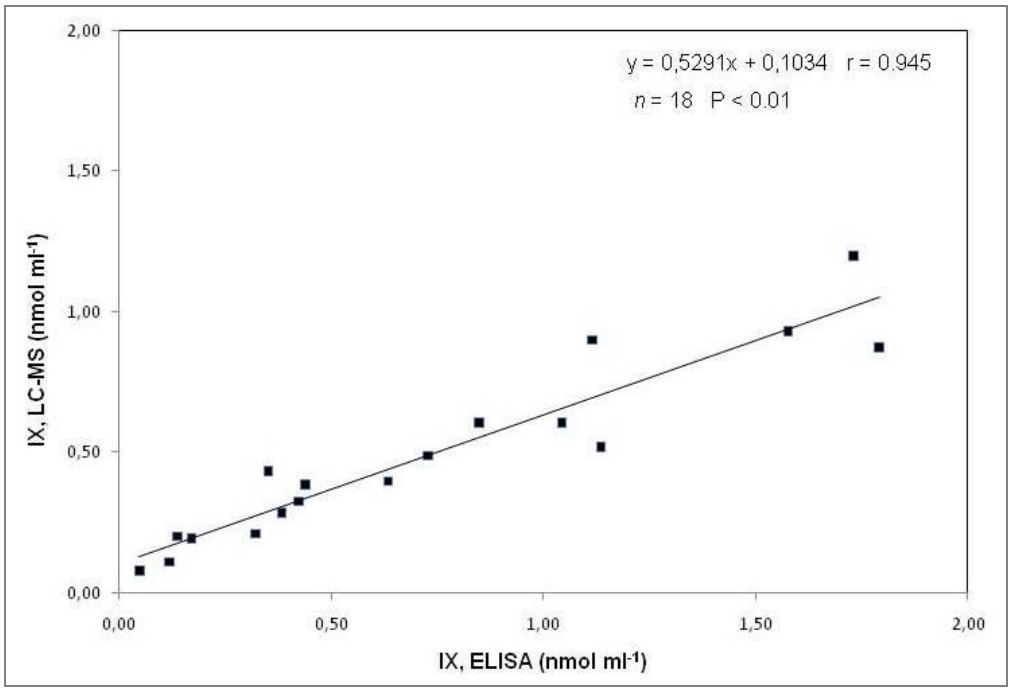
**Table 5.7:** Accuracy (%RE) in urine and serum at three QC levels (*n*=3).

| | URINE | | | SERUM | | |
|---|---|---|---|---|---|---|
| QC level | XN | IX | 8-PN | XN | IX | 8-PN |
| low | 9.2 | 14.1 | 11.9 | 4.9 | 2.3 | 10.8 |
| medium | 7.8 | 0.1 | 0.1 | 11.6 | 2.9 | 2.8 |
| high | 1.7 | 1.7 | 3.2 | 7.5 | 6.3 | 3.2 |

### *3.7 Correlation with LC-MS method*

We determined the correlation between the competitive ELISA and an established and validated LC-MS method described in Chapter 3 [150] by applying both methods on urine samples from a dietary intervention trial described in detail by Bolca *et al.* [130] (Chapter 4) and with Ethical Approval from the Ethics Committee of the Ghent University Hospital (EC UZG 2007/199; Belgian registration number: B67020072203; initial recruitment date: October 15, 2007). We selected 18 urine samples of subjects (both male and female, generally healthy individuals) who ingested with meals a hop-based supplement (MenoHop, Metagenics Europe): 1.20 ± 0.04 mg IX, 0.10 ± 0.01 mg 8-PN, 0.09 ± 0.01 mg 6-prenylnaringenin, and 2.04 ± 0.06 mg XN per capsule) three times a day during 5 days, after a run-in phase of at least 4 days. On the last day of the intervention spot urine samples were collected. The urine samples were extracted and hydrolyzed as described in section 2.11 and IX and 8-PN concentrations were measured with the competitive ELISA and compared with the results from LC-MS analyses. For the ELISA, values were based on four measurements with the final immunoassay and for LC-MS duplicate determinations were used. The correlations for both IX and 8-PN urinary concentrations were found to be highly significant: correlation coefficients were $r$ = 0.945 ($P$<0.01; $n$=18) and $r$ = 0.860 ($P$<0.01; $n$=18) for IX and 8-PN, respectively (Figure 5.4A and 5.4B). The correlation of urinary xanthohumol concentrations was not included in this study, because, first of all the levels in the patient samples were too low to get detected with the developed ELISA. Secondly, it has been reported before that xanthohumol has a very poor bioavailability [30, 65] and therefore this ELISA will not be directly applicable in the clinical field. Furthermore, serum correlations were also not investigated, because the amount of serum from the study was limited and the spiking of pooled blank serum would not have given an added value to our study.

**A)**




**B)**

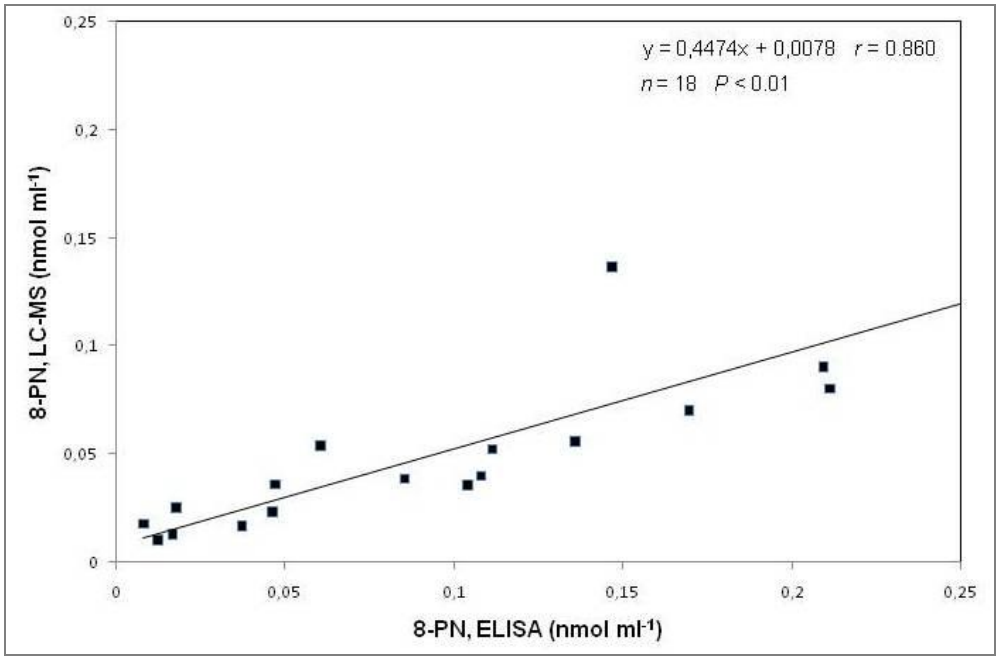



**Figure 5.4:** **Correlation graphs of significant correlation (Pearson's correlation coefficient) between urinary IX (A) and 8-PN (B) concentrations measured with the validated LC-MS method and ELISA.**

# 4. CONCLUSION

In conclusion, three monoclonal antibodies were successfully developed against the hop-derived prenylflavonoids XN, IX and 8-PN. They were integrated in an indirect competitive ELISA, which was validated for the measurement of the respective compounds in urine and serum samples. This ELISA technique requires a short sample preparation that consists of enzymatic hydrolysis and subsequent solvent extraction with diethyl ether, to reduce matrix effects and avoid potential over- or underestimation. The sensitivity of this assay, although acceptable, is not so high as with our established LC-MS method, as shown in a comparative table (Table 5.8), but can be further improved by changing the type of immunoassay. In addition, all three antibodies showed no substantial cross-reactivity with related compounds and both accuracy and precision of the measurements were within the criteria of acceptance. Therefore, this ELISA may serve as a reliable, simple and cost effective tool and as a promising alternative for chromatographic methods in intervention trials that require the assessment of the exposure to hop-derived prenylflavonoids. Additionally, these monoclonal antibodies may be valuable for other applications such as time-resolved fluoroimmunoassay, immunocyto- and histochemistry to reveal molecular mechanisms of XN, IX and 8-PN and the subcellular localization and tissue distribution of these compounds.

**Table 5.8:** Comparative table of indirect competitive ELISA and LC/APCI-MS method.

| Parameter | Indirect competitive ELISA | LC/APCI-MS method |
|---|---|---|
| **Sample prep** (after hydrolysis of conjugates) | urine and serum: LLE (diethyl ether) | urine: LLE (diethyl ether)<br>serum: SPE (C18) |
| **LOD** | <br>XN: 14.5 ng/mL<br>IX: 5.6 ng/mL<br>8-PN: 4.4 ng/mL | urine/serum:<br>XN: 0.6/1.4 ng/mL<br>IX: 0.2/1.5 ng/mL<br>8-PN: 0.4/1.5 ng/mL |
| **LOQ** | <br>XN: 41.0 ng/mL<br>IX: 13.9 ng/mL<br>8-PN: 17.1 ng/mL | urine/serum:<br>XN: 1.9/4.8 ng/mL<br>IX: 0.8/5.0 ng/mL<br>8-PN: 1.2/4.8 ng/mL |
| **Accuracy (%RE)** (mean of three QC levels) | urine/serum:<br>XN: 6.2/8.0<br>IX: 5.3/3.2<br>8-PN: 5.1/5.6 | urine/serum:<br>XN: 10.4/5.1<br>IX: 3.3/10.6<br>8-PN: 10.1/7.4 |
| **Precision (%CV)** (mean of three QC levels) | Inter-assay (urine/serum):<br>XN: 2.3/8.9<br>IX: 6.2/6.1<br>8-PN: 7.2/6.1<br>Intra-assay (urine/serum):<br>XN: 1.9/1.4<br>IX: 2.4/1.6<br>8-PN: 0.7/2.4 | Inter-assay (urine/serum):<br>XN: 16.3/10.1<br>IX: 10.7/12.4<br>8-PN: 14.4/9.7<br>Intra-assay (urine/serum):<br>XN: 7.1/5.2<br>IX: 5.2/5.6<br>8-PN: 6.0/3.9 |

# CHAPTER 6

## Application of a monoclonal antibody against xanthohumol

### IMMUNOCYTOCHEMISTRY AND -PRECIPITATION

This chapter was published as:

*The prenylated chalcone xanthohumol associates with histones in breast cancer cells – a novel target identified by a monoclonal antibody.*

Wyns, C., Van Steendam, K., Vanhoecke, B., Deforce, D., Bracke, M. and Heyerick, A.

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# 1. INTRODUCTION

Xanthohumol (XN) (3'-[3,3-dimethyl allyl]-2',4',4-trihydroxy-6'-methoxychalcone), a yellowish prenylated chalcone, was first isolated in 1913 by Power *et al.* [191] from the resin of hops (the female inflorescences of the hop plant (*Humulus lupulus* L.)) and later identified as a new natural chalcone by Verzele and co-workers [192]. It is the most important prenylflavonoid in hop (from 0.1 up to 1% (w/w)) and is, therefore, present in beer and hop-derived dietary supplements targeting the relief of menopausal symptoms [65]. Nevertheless, in beer, the concentration of xanthohumol is low (*e.g.* 28 µg/L in European style pilsner), due to low solubility and thermal isomerisation to the more soluble prenylflavanone isoxanthohumol during the brewing process [18]. Isoxanthohumol is a pro-estrogen as it can be further converted into the potent phytoestrogen 8-prenylnaringenin by demethylation effected by liver enzymes or intestinal gut bacteria as described by Nikolic *et al.* [19] and Possemiers *et al.* [52]. Hops are mainly used as a flavoring agent in beer, but over the last decade the health beneficial properties of hop derived terpenophenolics have gained increased attention, including the possible use of the prenylflavonoid XN as a chemopreventive agent. The news that the drinking of beer could have positive effects on mutagenesis and DNA adduct formation triggered the start of the search for possible constituents in beer with chemopreventive actions [193]. Miranda *et al.* [194] initiated the research into the anti-cancer bioactivity of XN, by screening it in a number of human cancer cell lines. XN was further described to inhibit metabolic activation of procarcinogens through cytochrome P450 enzymes (Phase I) [66, 67] and also to induce Phase II enzymes which can help in the detoxification of carcinogens [68, 69]. Gerhauser *et al.* [56] confirmed the broad-spectrum chemopreventive activity of XN, acting by multiple mechanisms at the stages of initiation, promotion and progression of cancer. In particular, besides the Phase I and II modulation of carcinogen metabolism, they established that the anti-inflammatory properties of XN by inhibition of cyclooxygenase (COX) enzymes also contribute to the potential antitumor-promoting mechanisms of the molecule. Vanhoecke *et al.* [195] described the anti-invasive properties of XN *in vitro* with breast cancer cell lines, mediated by the upregulation of the E-cadherin/catenin complex. Additionally, the prevention of

oxidative damage by scavenging reactive oxygen species (ROS) [196] and nitrogen oxide (NO) production [56, 71], but on the other hand also potential prooxidative properties of XN may trigger the antiproliferative mechanism and the induction of apoptosis [197, 198]. Obviously, in the area of cancer prevention, this chalcone has already been extensively studied and proved to be a particularly interesting molecule, at least *in vitro*. Furthermore, XN can serve as an anti-microbial and -viral agent [199-201], promote the osteogenic differentiation [203], ameliorate metabolic disorders [95, 204, 205] and has anti-inflammatory capacity. The latter can be mediated through nuclear transcription factor 2-antioxidant responsive element (NrF2-ARE) signaling [206], or by the inhibition of the activation of transcription factor nuclear factor-kB (NF-kB) [207] and inhibition of the expression of NF-kB dependent proinflammatory genes [208, 209]. Any possible therapeutic application is highly dependent on the overall bioavailability of XN. Caco-2 cell experiments showed that, although the intestinal absorption of XN is extremely low, it accumulates in the cytosol of intestinal cells due to high protein binding [30]. Its low intestinal absorption is also reflected in the urinary excretion after oral administration, which tends to be very low and seems to be dose dependent [31, 208]. Furthermore, XN is susceptible to microbial degradation and transformation in the intestine, *in vitro* experiments also showed glucuronidation and sulfatation in the liver as well as in the intestine and cytochrome P450 enzymes are responsible for its degradation [39, 211-213]. Although several molecular working mechanisms have been suggested and most of the publications have described downstream effectors, especially the characterization of the primary molecular target of XN is an underexplored field of research. The aim of the present study was to investigate the cellular localization of XN and to identify possible intracellular binding partners in a human breast cancer cell line (MCF-7/6). For this purpose a recently developed specific monoclonal antibody against xanthohumol was used in combination with immunocytochemical and -precipitation techniques.

## 2. EXPERIMENTAL

### *2.1 Chemicals*

Xanthohumol (XN) was available in our laboratory [52] and the mouse anti-xanthohumol $IgG_1$ antibody was an antibody manufactured in-house as described previously in Wyns *et al.* [214] and the previous Chapter 5. BSA, *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), 4',6-diamidino-2-phenylindole (DAPI), Tween-20 and Nonidet P-40 were purchased from Sigma-Aldrich (St. Louis, MO, USA). PBS, Tris-buffered saline (TBS), trypsin/EDTA, foetal calf serum (FCS), DMEM, HAM's F12, NuPage lithium dodecyl sulphate (LDS) sample buffer, penicillin, streptomycin, goat anti-mouse IgG Alexa 594 and Sypro Ruby Protein gel staining were supplied by Invitrogen (Carlsbad, CA, USA) and amphotericin B by Bristol-Meyers Squibb (Brussels, Belgium). NaCl, Tris and 4-hydroxybenzophenone (4-HBPH) were purchased from Acros Organics (Morris Plains, NJ, USA) as well as all chemicals and solvents (all of analytical reagent grade) for the analytical work. A 2D Quant Kit for protein concentration quantification was purchased from GE Healthcare (Uppsala, Sweden) and Protein G agarose for immunoprecipitation was obtained from Pierce (Rockford, IL, USA). Modified sequence grade trypsin (porcine) was purchased from Promega (Madison, WI, USA). Other materials and equipment for gel electrophoresis were obtained from Bio-Rad (Hercules, CA, USA). The solvents for the liquid chromatography were all of LC-MS quality (Biosolve, Valkenswaard, The Netherlands).

### *2.2 Cell culture*

The human mammary adenocarcinoma (MCF-7/6) cell line (from a pleural effusion of mammary adenocarcinoma), was kindly provided by Dr. H. Rochefort (Unité d' Endocrinologie Cellulaire et Moleculaire, Montpellier, France). This subline differs from the parental MCF-7 cell line because it has a nonfunctional E-cadherin complex and can express non-muscle myosin IIA [215]. These cells were grown and cultured in a 50/50 (v/v) mixture of DMEM/HAM's F12 with 10% heat-inactivated FCS, 100 IU/mL penicillin, 100 µg/mL

streptomycin and 2.5 µg/mL amphotericin B. The cells were incubated in a 100% water-saturated atmosphere with 10% $CO_2$ at 37 °C.

### *2.3 Quantification and stability of XN in cell culture medium via HPLC-UV*

The overall solubility of XN after supplementation to the cell culture medium was investigated, as well as the stability of XN in the medium at the culturing temperature of 37 °C. Growth medium (at 37 °C) was spiked with a solution of XN in 100% ethanol, so that final concentration of 10 µM was reached. Reference samples, spiked with a known amount of XN in ethanol and also blank samples (medium without XN) were included. The culture media, spiked with XN were kept at 37 °C for different time periods (30 min, 1 h, 2 h, 4 h, 6 h and 24 h). Each time point was tested in triplicate. Afterwards, all the samples were spiked with 100 µL of internal standard (4-HBPH; 1 mg 4-HBPH in 10 mL MeOH) and extracted three times with ethyl acetate. The organic solvent layers were evaporated under a gentle nitrogen stream. The resulting residue was dissolved in 500 µL MeOH/$H_2O$ (50/50) + 0.025% formic acid and 20 µL was injected in the HPLC for analysis. For the chromatography a Waters Alliance 2695 Separation Module (Waters, Milford, MA, USA) equipped with a Waters 996 DAD as reported by Possemiers *et al.* [52]. Detection was done simultaneously at 370 nm for the quantification of XN and at 295 nm for the internal standard. Data processing was performed with the Waters Millenium 3.2 software.

### *2.4 Cellular uptake of XN in MCF-7/6 cells*

MCF-7/6 cells were seeded in 6-well plates (Nunc, Roskilde, Denmark) at a density of 5 x $10^4$ cells/mL on day one. On day three, half of the medium was aspirated and fresh medium spiked with XN (dissolved in 100% EtOH) was added, so that the final concentration in the well was 10 µM. The cells were incubated for different time periods (30 min, 1 h, 2 h, 4 h, 6 h and 24 h). A blank control (100% EtOH without XN) was included in each experiment and each time point was set up in six replicates. After the end of the treatment period, the

medium was aspirated and transferred to a tube. Then, each well was washed twice with 1 mL of medium and each wash was collected in the respective tube. After three further washing steps with 1 mL PBS, 100 µL lysis buffer (150 mM NaCl - 1% Nonidet P-40 - 50 mM Tris-HCL; pH 7.4) was added to each well and the plates were placed on ice for 30 minutes. After lysis, the cells were scraped of the plate and washed with 900 µL PBS and the resulting lysates were frozen at -20 °C until analysis. Before analysis, the collected medium from the wells and the lysates were all spiked with 100 µL internal standard and subjected to a solvent-solvent extraction with ethyl acetate that was repeated three times. The pooled organic solvent from the triple extraction step was dried under a gentle nitrogen stream and the residue was dissolved in 200 µL methanol. 20 µL of each sample was analyzed with HPLC-UV as described in section 2.3.

## *2*.5 Immunocytochemistry

Round glass coverslips were placed in a 24-well plate and MCF-7/6 cells were seeded as a suspension with a density of 3.5 x $10^4$ cells per mL on day one. On day three, half of the medium was replaced by growth medium supplemented with XN (dissolved in 100% EtOH) or an identical volume of 100% EtOH (blank), so that a final concentration in the well of 10 µM XN was reached. The ethanol concentration in the wells was 0.35 % (v/v) for every condition. Treated cells were incubated for different time periods at 37 °C (30 min, 1 h, 2 h, 4 h, 6 h and 24 h). During the treatment the cells were also visualized microscopically to monitor overall cell health. After aspiration of the culture medium the cells were washed twice with PBS (37 °C) and fixed for 20 min in 3% paraformaldehyde in PBS at room temperature. After fixation, the coverslips were washed in PBS and cells were permeabilised with 0.2% Triton-X after 10 min incubation with $NH_4Cl$. Then, after three washing steps with TBS, the fixed cells were blocked with 5% BSA in TBS for 30 min at room temperature. After the blocking step, blanks and positive controls were incubated with primary antibody, *i.e.* anti-xanthohumol antibody, overnight at 4 °C. The negative control (cells treated with 10 µM XN for 6 h) was incubated with an isotype-matched irrelevant mouse $IgG_1$ antibody. The next day, after three washing steps, the coverslips were incubated with goat anti-mouse Alexa

594 antibody for 90 min at room temperature, and 5 min before the end samples were counterstained with DAPI (400 ng/mL). After washing, the coverslips were mounted in glycergel (Dako, Glostrup, Denmark) on a microscopic slide and kept in the dark at 4 °C until evaluation. The immunocytochemical localization was optically verified with a Leitz Dialux 20 microscope (Leica, Wetzlar, Germany) using the NPL Fluotar 23 50/1.00 oil immersion objective. Afterwards, confocal images were captured with a Leica Sp5 AOBS confocal microscope (Leica, Mannheim, Germany). Images were taken using a 63x HCX PL Apo 1.4 oil objective. DAPI was excited with a UV diode laser at 405 nm and Alexa 594 with a HeNe Laser at 543 nm. Z-sections were made with stepsize 0.5.

### *2.6 Sample preparation for immunoprecipitation*

MCF-7/6 cells were seeded in culture flasks (25 $cm^2$) and when the cells reached approximately 70-80 % confluency, they were treated with XN for 2 h at a final concentration of 10 µM. Blanks treated with ethanol (0.35% (v/v)) served as a negative control. After the treatment, the medium was aspirated and the cells were washed twice with ice-cold PBS, then the cells were lysed on ice for 30 min with a non-denaturing lysis buffer (see section 2.4 for composition) with a complete protease inhibitor cocktail (Roche, Penzberg, Germany). The cells were scraped from the flasks and the supernatant of the centrifuged lysates was stored at –20°C. The protein concentration of the lysates was determined using a 2D Quant Kit (Invitrogen, Carlsbad, CA, USA).

### *2.7 Immunoprecipitation with an anti-XN antibody*

The isolation and concentration of possible target proteins of XN was performed by immunoprecipitation (IP) with Protein G and a specific in-house monoclonal anti-XN antibody. In particular, 40 µL settled resin (with Protein G cross linked to agarose) was washed twice with 500 µL lysis buffer. Lysates (500 µL) were pre-incubated under slow agitation with 100 µL antibody (1 mg/mL) for 1 hour at 37 °C, and then added to the beads. The samples with the beads were placed on a rocker overnight at 4 °C. The next day, after 5

washing steps with 500 µL cold lysis buffer and collection of all the supernatants, the pellet of beads with bound proteins was recovered and saved for one-dimensional gel electrophoresis.

### *2.8 One-dimensional gel electrophoresis (1D-PAGE)*

The samples from the IP, the supernatants from the washing steps in the IP and the original cell lysates were denatured with 40 µL NuPage LDS sample buffer supplemented with 4 µL of freshly made 1 M DTT and incubated at 90 °C for 5 minutes. After centrifugation, the denatured and reduced protein samples were loaded on a 15 or 18% Tris-HCl polyacrylamidegel (Biorad, Hercules, CA, USA) and electrophoresis was performed by applying 150 V for 30 minutes, followed by 200 V for one hour. The gel was fixed for 30 minutes in a 10% MeOH, 7% acetic acid solution and afterwards the proteins in the gel were stained overnight using Sypro Ruby Protein Gel staining (Invitrogen). Protein patterns were scanned and digitized using the VersaDoc Imaging System (Biorad).

### *2.9 Western Blotting*

The 18% gel and nitrocellulose membranes (Bio-Rad) were incubated for 15 minutes in CAPS buffer (pH 11) with 20% MeOH and electrophoretic transfer of proteins was performed by tank blotting in a Trans Blot Cell (Biorad) with CAPS buffer at 120 V for 110 minutes. Successful protein transfer was checked using Ponceau S solution staining (Sigma).

### *2.10 Protein identification by LC-MS/MS (Q-TOF)*

The in-gel porcine trypsin digestion of the proteins of interest from the IP was performed as described earlier [216]. Briefly, gel pieces were excised from the gel and washed twice in 50% ACN in 50 mM ammonium carbonate for 10 min. After reduction with 10 mM DTT at 56 °C for 10 min and alkylation with 100 mM iodoacetamide for 45 min at room temperature (both in 50 mM ammoniumcarbonate), and succeeding washing steps,

the pieces were dehydrated with MeCN using a Speed-Vac concentrator (Eppendorf, Le Pecq, France). Then, they were rehydrated with 10 ng/µL sequence grade modified trypsin for 30 min on ice. After digestion overnight at 37 °C the liquid was collected and the resulting peptides were extracted with 50% ACN followed by an extraction with 100% ACN. The pooled extract was finally completely dried using the Speed-Vac concentrator. Prior to analysis, peptides were resuspended in $H_2O$ + 0.1 % formic acid and were then analyzed and identified by LC-MS/MS, using a Q-TOF Ultima Mass spectrometer (Waters). The data were processed using Mascot Distiller and searched against the Swissprot human database, using the in-house Mascot daemon searching algorithm. Identification was considered positive with a *P*-value < 0.05.

### *2.11 Histone H2A immunodetection*

The blot was blocked with 1% BSA in 0.3% Tween-20 in PBS for one hour and incubated with histone H2A rabbit anti-human polyclonal antibody (LifeSpan Biosciences, Seattle, WA, USA) at a concentration of 1/1000 in blocking buffer. Overnight incubation with the primary antibody was followed by three washing steps (5 minutes) and incubation with HRP labeled goat anti-rabbit IgG (1/20 000; Pierce) (1 hour). The ECL detection was carried out using Supersignal West Dura Extended Duration Substrate (Pierce) and protein patterns were scanned and digitized using the VersaDoc Imaging System (Biorad).

# 3. RESULTS

## *3.1 Solubility and stability of XN in in vitro conditions*

First, we investigated the solubility of XN in the culture medium under the conditions of cell culture. Xanthohumol is a rather apolar molecule, with very poor solubility in aqueous media, but dissolves well in ethanol and DMSO [65]. Since the organic solvent concentration in cell culture should be kept as low as possible, it was necessary to check for possible solubilisation problems that could arise for XN during the experiments. Although the concentration of XN is quite low during our experiments (10 µM) we performed this investigation since good dissolution had to be assured. An extraction procedure for XN from cell culture medium was optimized and the final method existed of three solvent-solvent extraction steps of spiked medium (three concentrations) with ethyl acetate by intensive vortex mixing. The recovery of XN from the medium was for the 10 µM concentration respectively 91.8% (± 7.9 %). This extraction method was also used in the stability experiment with XN (10 µM incubated for 30 min, 1 h, 2 h, 4 h, 6 h and 24 h). The recovery of XN from the growth medium for all time periods was good with an average of 83.3 (± 10.0%) recovery for 10 µM was obtained, respectively. No detectable degradation products were observed using HPLC-UV.

## *3.2 Measurement of the uptake of XN in MCF-7/6 cells*

XN was added to the medium of the MCF-7/6 cells at a concentration of 10 µM and the cellular uptake was estimated by measuring the remaining concentration of XN in the medium at the end of each incubation period (Figure 6.1). The quantification of XN in the collected media from this experiment revealed that already after 30 minutes about 17.8% (± 1.49 %) of the original level XN has associated with the cellular fraction. This situation remains more or less stable until after 4 h, when 26.7% (± 1.5 %) uptake is reached. After 6 h, about half of the original amount of XN can be retrieved in the medium of the cells and this does not change until the 24 h time point and end of the experiment. Figure 6.1 also

shows the concentration of XN measured in the cell lysates from the uptake experiment. A slight increase in XN concentration over time is noticeable, but even the sum of these and the medium fractions, does not lead to the total dose of the administered XN. Hence, there must be another reason, *e.g.* loss during sample preparation, association with non-extractable cellular material or rapid biotransformation, for the discrepancy between XN concentrations in the medium and those in the cell fractions.

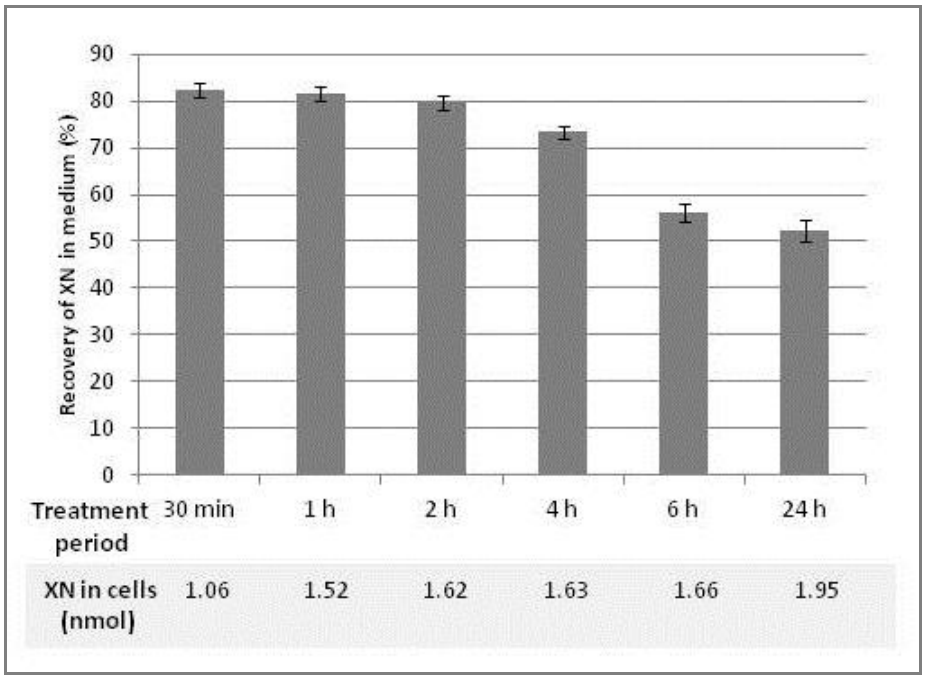


**Figure 6.1:** **Recovery of XN (10 µM) in medium of MCF-7/6 cells and amount of XN retrieved in cells, after different incubation periods (*n*= 6; mean ± SD).**

## *3.3 Immunocytochemical localization of XN in MCF-7/6 cells*

Through indirect immunofluorescence we investigated the subcellular distribution of XN inside MCF-7/6 cells. First of all, an optimal staining procedure had to be obtained for this experiment and several factors, including fixation method of the cells, concentrations of primary and secondary antibodies, temperatures and incubation periods, were optimized. Finally, an optimized and reproducible procedure, described in section 2.5, was obtained.

Preliminary experiments revealed that after a treatment period of 6 h with 10 µM XN, staining of XN in the cells could be obtained. A particularly granular pattern was observed with a high nuclear density, while a slight staining could be observed in the cytoplasm, with no specific organelle or cellular structure highlighted. The blank (vehicle control, stained with anti-XN antibody and secondary antibody) and the negative control (treatment with XN 10 µM 6 h, stained with unlabeled irrelevant mouse $IgG_1$ and secondary antibody) gave no staining at all (Figure 6.2a+b). Furthermore, the same optimized staining procedure was repeated at least five times on cells that were treated for 30 min, 1 h, 2 h, 4 h, 6 h and 24 h with 10 µM XN. As can be observed in Figure 6.2a, already after 30 min the same pattern as with the 6 h treatment can be observed, although with a weaker staining. Treatment with a concentration of 50 µM XN for the same time periods gave similar results (pictures not shown here). Confocal imaging showed that with increasing treatment period, the nuclear staining pattern only intensified and also emphasized that there was no staining of the nucleoli. These findings provided us with the information that XN was widely distributed in the nucleus. Nevertheless, several localizations like cytoplasmic and nuclear membranes, cytoskeletal structures, and certain organelles (*e.g.* ER, mitochondria…) could be excluded based on the typical granular and mainly nuclear pattern.

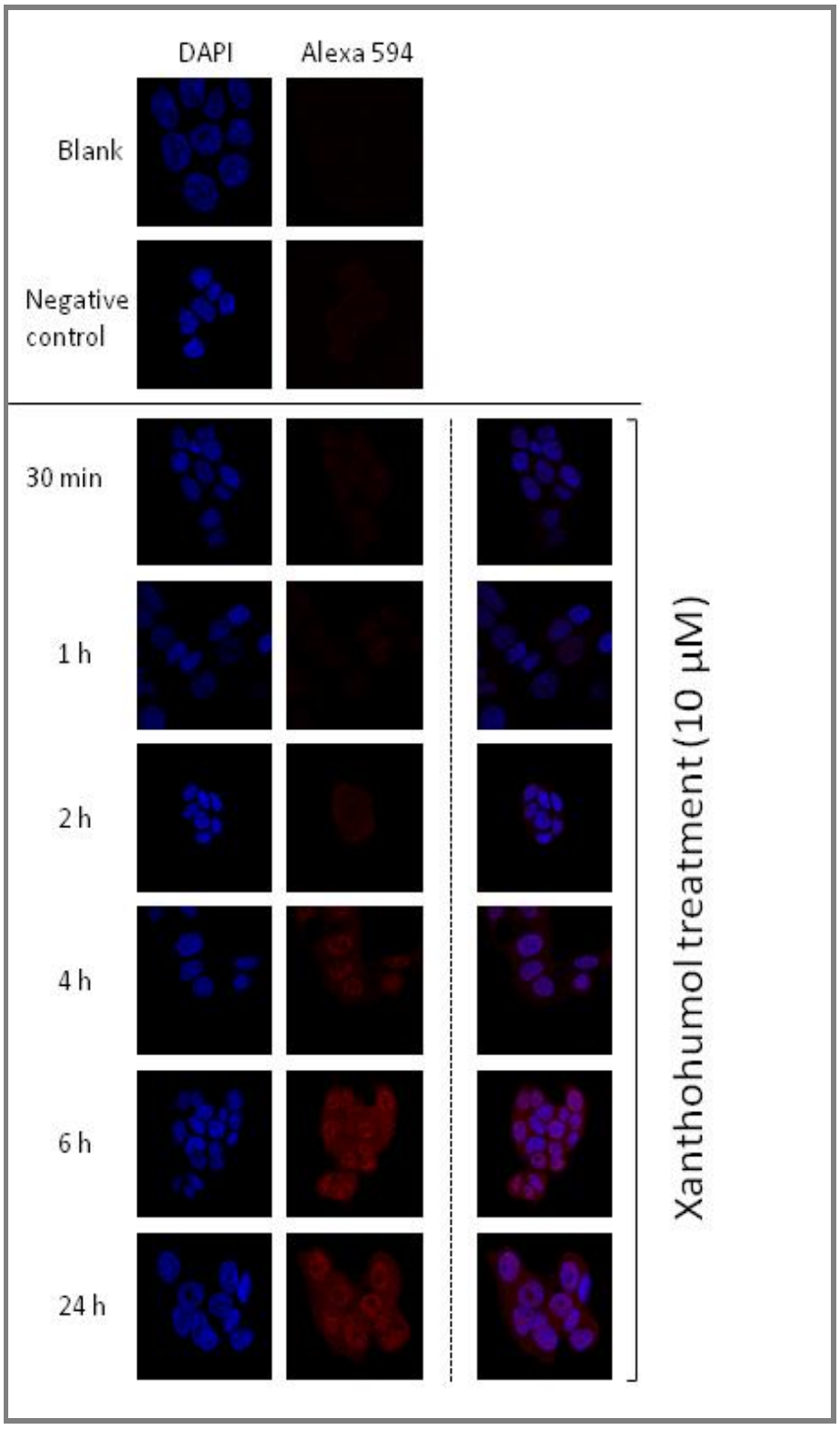


**Figure 6.2a:** **Immunostaining of XN with anti-XN antibody in MCF-7/6 cells. Nucleus stained with 4,6-diamidino-2-phenylindole (DAPI) and anti-XN antibody with Alexa 594 anti-mouse IgG (blank: no XN; negative control: XN and mouse $IgG_1$ as primary antibody).**

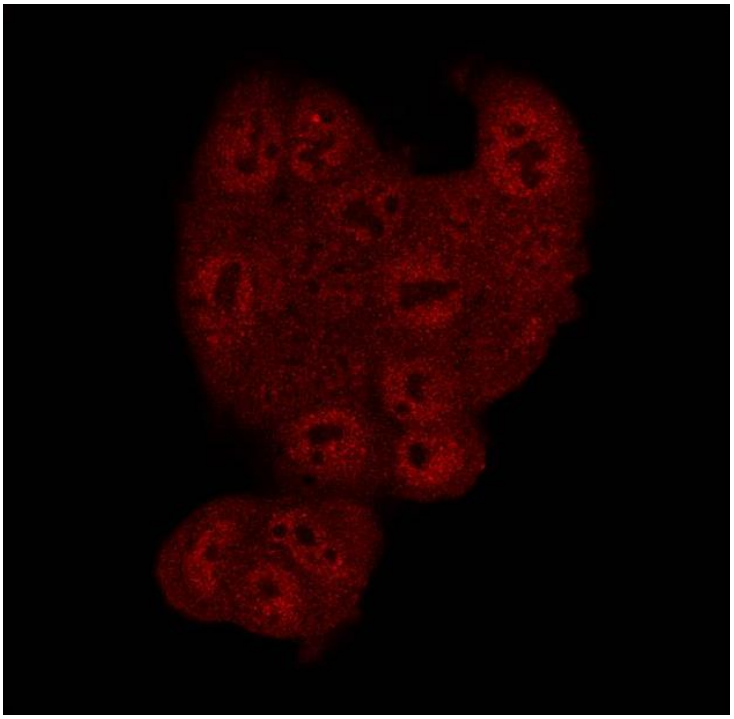

**Figure 6.2b:** zoomed in on Alexa-staining of XN (positive control; 10 µM 6 h treatment).

### *3.4 Identification of target binding proteins of XN in MCF-7/6 cells*

Immunoprecipitation with an anti-XN antibody was used to isolate possible target proteins of XN in MCF-7/6 cells. The proteins from the lysates after treatment (10 µM XN for 2 h) that were retained by immunoprecipitation were subjected to 1D-PAGE. As depicted in Figure 6.3, this resulted, for the treatment group, for the 10 µM treatment 2 h time point in clear positive staining of a series of proteins in the low-molecular weight area below 20 kDa. Immunoprecipitation of untreated cells (6 h, vehicle control) did not produce the same pattern of protein bands in that area. Subsequently the relevant proteins were further identified after in gel digestion. Mass spectrometric analysis revealed that the bands of interest from the treatment group were belonging to the histone protein families. More specifically, histones H2A, H2B and H4 with molecular weights of 14 kDa (H2A and H2B) and 11 kDa, respectively (which corresponds to their location on the gels) were identified (all with $p$-value < 0.05). In order to confirm the identity of one member of these protein family, immunoblotting with anti-histone H2A antibody was performed.

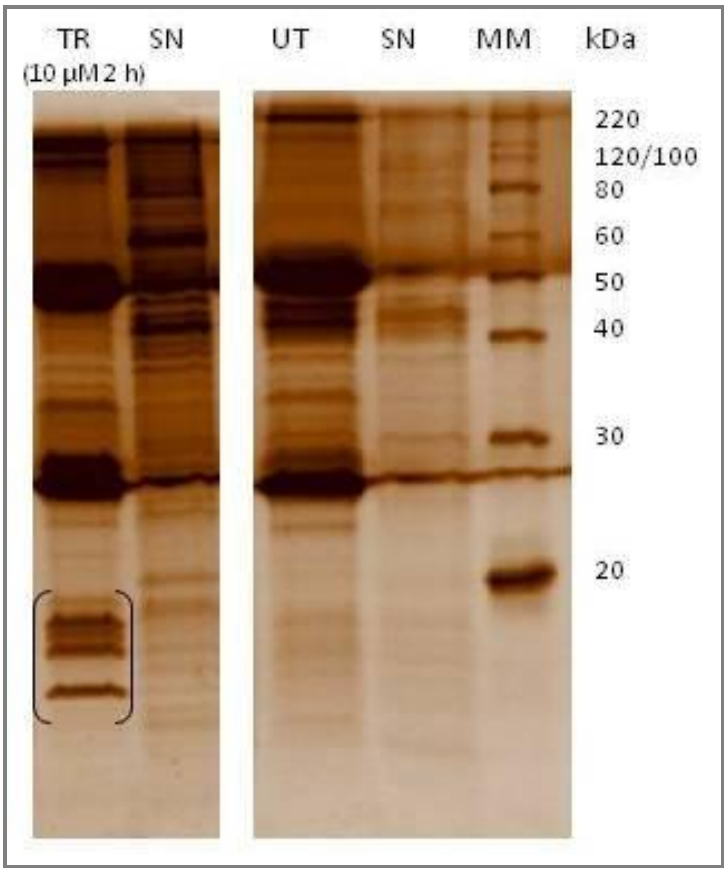


**Figure 6.3:** **1D-PAGE of: lysates from untreated (UT) MCF-7/6 cells after isolation with IP; detection of histones H2A, H2B and H4 in cell lysates after incubation with XN (10 µM 2 h) after isolation with IP. The proteins (below 20 kDa and between brackets) were identified by mass spectrometry. Next to each lane with the isolated proteins from IP, the supernatants (SN) from that IP was spotted, a molecular marker (MM) was used for indication of the molecular weights.**

## *3.5 Immunodetection of histones in MCF-7/6 cell lysates*

One of the cell lysates (10 µM XN, 2 h treatment) in which, after immunoprecipitation, histones were identified as the binding proteins of XN *in vitro* (section 3.3), was subjected to immunodetection with an anti-histone H2A antibody. The results of this control experiment are summarized in Figure 6.4. In the lane of the untreated cell lysate that also underwent immunoprecipitation, no histone H2A could be detected. On the other hand, in the treated cell lysate one of the bands below 20 kDa was identified as histone H2A. Histone H2A was also identified in cell lysate before immunoprecipitation, although with a lower intensity.

Thus, immunoprecipitation with the anti-XN antibody resulted in an enrichment of histone H2A in our sample.


**Figure 6.4:** **Western blot with immunodetection with anti-histone H2A antibody. Lane 1: untreated cell lysate after IP, lane 2: treated cell lysate after IP (10 µM 2 h) and lane 3: treated cell lysate (10 µM 2 h) without IP. In lane 2 and 3 visualisation of histone H2A at 14 kDa.**

## 4. DISCUSSION

This study aimed to gain insight into the *in vitro* uptake and the subcellular fate of XN in a breast cancer cell line (MCF-7/6) and to identify possible intracellular binding partners to provide more information about the underlying mode-of-action of bioactivities reported for XN. Initially, we studied the solubility and stability of XN in cell culture medium at experimental conditions, an aspect often forgotten in molecular studies that can greatly influence the results. It was shown previously that XN does not readily dissolve in aqueous solutions and presumably is neither so stable in it [18, 217]. Our experiments showed that at 37 °C XN dissolves as good as completely in the culture medium and the stability is also satisfactory. Only after 6 h of incubation about 25% of the original amount of XN could not be retrieved, but after 24 h this was still the case. We concluded that the solubility and stability of XN in our cell culture medium were appropriate for further experiments. The differences in solubility and stability of XN with previous reports may be due to the nature of the growth medium, which differs from water and may cause cosolvation or contain certain lipophilic components that enhance dissolution and stability. Motyl *et al.* [218] recently investigated the solubility of xanthohumol in cell culture work and concluded that a minimum of 10% serum in the medium increases the solubility and prevents the absorption to various plastic materials. They also assumed possible interactions with serum proteins in *in vitro* experiments, which may impact upon the uptake of xanthohumol in the cells.

Additionally, we investigated the cellular uptake of XN in MCF-7/6 cells *in vitro*. Already after 30 minutes a significant portion of XN was associated with the cellular fraction and after 6 h about 45% of the total amount of XN added could not be retrieved from the medium fraction. We also observed that, although the added amount of XN tends to decrease rapidly, this trend slows down afterwards (1 h and 2 h). This observation may be due to association with the cell wall, rather than an effective uptake, but could not be clarified from this type of experiment. After 24 h, approximately the same amount is

recovered from the medium as with 6 h, which may indicate that after a rapid and strong accumulation in the cells a saturation plateau is reached. Pang *et al.* [30], who studied the uptake of XN in intestinal cells, observed a strong intracellular accumulation of XN. Through ultrafiltration and LC-MS techniques they provided evidence that XN bound to cytosolic proteins in Caco-2 cells treated for 1 h with 10 µM XN. Furthermore, covalent binding of XN with cystein residues of proteins like Keap 1 by a Michael-Type addition has been reported as a possible first step in the mechanism of inducing chemopreventive activity [219].

Our immunocytochemistry experiments showed that XN can rapidly locate intracellularly, both cyto- and nucleoplasmic, but tends to accumulate more in the nucleus over time in comparison to the cytoplasm. After 6 h exposure to a 10 µM concentration of XN, resulted in a clear nuclear staining, with a granular pattern. On the other hand, the faint, typically aspecific, staining in the cytoplasm that already appeared after 30 min did not further increase in intensity to the same extent as the nuclear staining. These observations, which were highly reproducible, instructed us to isolate/purify the binding partner of XN in conditioned cell lysates of MCF-7/6 cells by means of immunoprecipitation. Using Q-TOF mass spectrometry we were able to identify histones (*i.e.* histone H2A, H2B and H4) as the proteins that were differentially detected on the 1D-PAGE of immunoprecipitated XN-positive cell lysates. The identity of one of the bands of interest in the low-molecular region (below 20 kDa) was confirmed by means of Western Blotting and immunodetection. The application of our specific monoclonal antibody against the prenylated flavonoid XN, opens up a whole field of research opportunities related to the unraveling of the exact mode-of-action of XN. To the best of our knowledge, histones have not been reported previously as possible protein targets of XN in breast cancer cells.

Histones are very basic proteins that form an octameric core, consisting of a H3-H4 tetramer and two H2A-H2B dimers, around which the DNA strand is coiled. The modification of histone proteins is an epigenetic mechanism that effects the chromatin structure and

gene expression. Acetylation for instance, one of the posttranslational modifications, which is regulated by opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs), affects the function of transcription factors and the subsequent oncogenic transformation. Epigenetic regulators, influencing chromatin modifications at the histone core, have been under investigation in recent times, as promising therapeutics in cancer therapy. Dietary polyphenols, which have been suggested as potential chemopreventive agents, are also thought to modulate epigenetic alterations in cancer cells, but the precise mechanism how they regulate and effect modifications is hardly unraveled. Link *et al.* recently [220] reviewed the role of polyphenols like curcumin (curry), (-)-epigallocatechin-3-gallate (EGCG; green tea) and several others which can indirectly induce histone modifications acting as HAT and HDAC inhibitors. It would be interesting to investigate if the introduction of those inhibitors into the type of experiment we performed, would impact upon xanthohumol binding on histones. Methylation and demethylation of proteins, on the other hand, plays also an important role in nuclear hormone receptor regulated transcription. Xanthohumol, to the best of our knowledge, is not known for its direct action on steroid receptors. In estrogen receptor dependent cell lines like MCF-7/6, xanthohumol may influence nuclear hormone signaling indirectly by interference in histone modification processes. Additionally, it would also be recommended to repeat experiments in steroid free conditions to clarify the possible role of xanthohumol in NR-mediated transcription.

Clearly, these are all assumptions based on preliminary findings and basic knowledge of nuclear signaling pathways. Hypothetically, in our case we could assume that the association of XN with histones, may interfere with the posttranslational modifications of histones and therefore explain its versatile spectrum of effects on cancer cells. Nevertheless, this interaction needs to be worked out in detail, *e.g.* what amino acids or specific sites are targeted, what kind of interaction is there between XN and histones, is it directly, and furthermore, is there a modulation of the gene expression accomplished. To check the universal character of this finding, other cell lines would have to be applied in this type of experiment. Additionally, medicinal chemistry approaches could be applied for the

optimization of derived molecules targeting the same application. Obviously, future studies are necessary to reveal the biological relevance of the binding of XN to histones and its consequences and the availability of a specific monoclonal antibody and the established, optimized techniques from this study has already opened new perspectives and may spur further research in this field.

# Summary and general conclusions

This thesis describes the development and validation of two major methods for the analytical determination of phytoestrogens in biological matrices, *i.e.* a chromatographic method coupled with (APCI-)MS for the determination of 13 phytoestrogens (including gut metabolites) in urine and serum; and a non-chromatographic method using monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA) for the hop-derived xanthohumol (XN), isoxanthohumol (IX) and 8-prenylnaringenin (8-PN). Furthermore, the focus also lies on the exploration of applications of both methods and tools in the analysis of real samples.

In Chapter 1, on the basis of a literature survey, the targeted compounds, *i.e.* phytoestrogens were discussed on different levels like food sources, activities, bioavailability and metabolism. Phytoestrogens are defined as plant-derived non-steroidal secondary metabolites, which can mimic or modulate the actions of endogenous estrogens usually by binding to estrogen receptors. The majority of phytoestrogens are flavonoids, substituted phenolic compounds, belonging to three groups, *i.e.* isoflavones (from soy (*Glycine max* (L.) Merr.), prenylated flavonoids (from hops (*Humulus lupulus* L.) and coumestans (from red clover, alfalfa). The non-flavonoid lignans (from flax seed (*Linum usitatissimum* L.) are another large group of phytoestrogens which can also be daily consumed through plant-based food stuff or through the intake of dietary supplements. Concomitantly, phytoestrogens have been in the spotlight as very interesting research subjects, because of their broad spectrum of activities in the prevention and protection against certain diseases like cancer, cardiovascular disease and osteoporosis. Their health properties are largely depending on their bioavailability and consequently the physiological levels that are reached after consumption. The intestinal metabolism has also shown to play an important role in the explanation of many inter-individual differences in exposure levels of hormonally active compounds. Nevertheless, to control the intake and uptake of phytoestrogens and their effects at cellular level, either in clinical environments or controlled laboratory conditions, analytical tools for their determination and quantification are required and useful tools.

In Chapter 2 of this thesis the aims and most important objectives are outlined. The latter were part of a contracted research on phytoestrogens from supplements and their biological availability, physiological and toxicological impact with respect to human health in food supplements (acron: FYTOES) supported by the Federal Public Service (Health, Safety of the food chain and environment.

Chapter 3 describes the successful development and optimization of a new analytical HPLC-APCI(MS) method for the determination and quantification of 13 phytoestrogens including their precursors and gut microbial metabolites (genistein (GEN), daidzein (DAID), equol (EQ), dihydrodaidzein (DHD), *O*-desmethylangolensin (*O*-DMA), coumestrol (COUM), secoisolariciresinol (SECO), matairesinol (MAT), enterolactone (ENL), enterodiol (END), xanthohumol (XN), isoxanthohumol (IX) and 8-prenylnaringenin (8-PN)) in urine and serum. The simple sample preparation procedure consists of enzymatic deconjugation followed by liquid-liquid extraction (LLE) or solid-phase extraction (SPE) for urine or serum, respectively.

During the method development, first of all, the chromatographic separation of the analytes of interest and internal standard was optimized, using reversed phase chromatography. Several factors like the stationary phase, mobile phase composition, pH, column temperature and mobile phase additives were optimized to ensure an adequate separation within a short run time. The final method uses an XBridge C18 column at a temperature of 55 °C with a mobile phase gradient consisting of water and a methanol/acetonitrile mixture (80:20, w/w), both acidified with 0.025% (v/v) formic acid. In 20 minutes the target compounds are separated with baseline resolution (except for EQ, GEN and ENL; $R < 1.5$) and the column is equilibrated for the next run. Subsequently, by coupling the LC to an Agilent 1200 series Multimode Source detector, complete resolution was accomplished. Next, for each component individually, the interface and MS parameters were optimized and the optimal ionization method was chosen. Atmospheric Pressure Chemical ionization (APCI), operating in the positive mode generated pseudomolecular ions $[M + H]^+$ for most compounds, except for SECO which gave $[M+H-2H_2O]^+$ as the base peak. Only END and ENL were ionized through APCI in the negative mode ($[M - H]^-$), because higher

sensitivity was obtained. The fragmentor voltage, which differs per molecule, was set using the chosen ionization method. Also four MSD signals were set, in view of sensitivity and resolution and finally, gain values and MSD cycle times were set per programmed signal. Furthermore, this bioanalytical method was fully validated (according to the recommendations of the FDA) on the basis of selectivity, linearity, sensitivity, accuracy and precision. As a result this method was found suitable for routine analysis of samples with phytoestrogen levels in the lower ppb range (LOD: urine: 0.2-7.7 ng/mL; serum: 1.4-20.4 ng/mL), with the exception of SECO (urine: 65.1 ng/mL; serum: 132.6 ng/mL), with linearity ($r^2 > 0.995$) for all the analytes over the dynamic ranges. It also proved to allow an accurate and precise quantification of the targeted phytoestrogens and their metabolites. The accuracy (expressed as percentage relative error %RE) was within the acceptable ranges of 20% and inter-assay precision for urine and serum did not exceed 20%. The intra-assay precision was below 10% for urine and serum for all analytes, except for END and DAID and ENL in urine and serum, respectively. Also an assessment of matrix effects in urine and serum was made following the guidance of Matuszewski et al. [110]. Overall, there was found no more than 15% variation due to matrix effects in urine and serum.

In Chapter 4 the developed and validated LC/(APCI)MS method served as a valuable tool in the analysis part of a randomized cross-over dietary trial with co-supplementation of soy-, hop-and/or flax-based food supplements by Bolca *et al.* [130]. This study revealed that the bioavailability of mixed phytoestrogens is strongly determined by the microbiome of each individual and personalized screenings prior the use of these combinations in supplements, are therefore highly recommended.

Chapter 5 gives an overview of the development of monoclonal antibodies against phytoestrogens and their subsequent application in an enzyme-linked immunosorbent assay (ELISA) for biological matrices. The aim was to develop monoclonal antibodies against daidzein, genistein, coumestrol, xanthohumol (XN), isoxanthohumol (IX) and 8-prenylnaringenin (8-PN). Several polyclonal antibodies have been developed in the past

against phytoestrogens like daidzein, genistein, equol, coumestrol, enterolactone [175-181] what proves their immunogenicity. Monoclonal antibodies, which have the advantages of their specificity, homogeneity and long term availability, are up till now only available against daidzein, genistein and equol. Although, it has to be kept in mind that before immunization the target phytoestrogens have to be conjugated to bovine serum albumin, because with their low molecular weight, they normally do not evoke immune responses *in vivo*.

In that way, the first step in the development was the coupling of the target compounds to BSA, forming 4'-*O*-carboxymethyldaidzein, -genistein, -xanthohumol, -isoxanthohumol, -8-prenylnaringenin and 3-O-carboxymethylcoumestrol. Secondly, after immunization of the BALB/c mice with the antigens (*i.e.* phytoestrogen-conjugates), by applying a cell fusion procedure with polyethylene glycol (PEG) to the mouse spleen cells and murine myeloma NS0 cells, the respective hybridomas were constructed. With HAT selection medium, that contains aminopterin which blocks the *de novo* pathway, the fused B cell-myeloma hybrids are distinguished from unfused myeloma and B-cells. Then, with a non-competitive screening ELISA based on antigen-coated plates, only a few hybridomas could be selected, moreover those which produced antibodies against XN, IX and 8-PN. The latter antibodies were then also tested in an indirect, yet competitive ELISA to investigate the affinity of the produced antibodies for the original compounds of interest. The monoclonality of the antibodies was then ensured via limiting dilution. The clones of interest were grown in CELLine bioreactors and large amounts of monoclonal antibodies could be harvested every month for some time. The monoclonal antibody solutions were purified from the medium using FPLC and HiTrap Protein G columns. In total 59 mg , 195 mg and 52 mg of pure (checked with gel electrophoresis and Coomassie blue staining) anti-XN, anti-IX and anti-8-PN were obtained.

Using purified monoclonal antibodies, the different steps and parameters of the competitive ELISA (dilutions for the coating antigen, the blocking buffer and the primary and

secondary antibody, and the type of secondary antibody and enzymatic substrate, as well as the incubation temperature and time periods) were optimized. The final ELISA procedure makes use of immunogen-coated microtiterplates and a peroxidase-labeled anti-mouse $IgG_1$ secondary antibody with ABTS as a chromogenic substrate. For XN the $IC_{50}$ value derived from the standard curve was 62.91 ng/mL, and for both IX and 8-PN 37.15 ng/mL. In cross-reactivity studies, the mAbs proved to possess highly specific binding capacities in an optimized competitive indirect ELISA.

The assay was validated for the quantitative analysis of XN, IX and 8-PN in urine and serum. A simple sample pretreatment procedure using a diethyl ether extraction was optimized and the recoveries and matrix effects were assessed. The validity of the established assay was tested and mean inter- and intra-assay variations in urine were 2.32% and 1.91%, respectively for XN, 6.24% and 2.39%, respectively for IX and 7.18% and 0.74%, respectively for 8-PN. In serum, the mean inter- and intra-assay variations were 8.90% and 1.37%, respectively for XN, 6.13% and 1.57%, respectively for IX and 6.13% and 2.43%, respectively for 8-PN. Furthermore, the method demonstrated excellent accuracy and significant correlation with measurements by the established and validated HPLC-MS method from Chapter 3.

Chapter 6 deals with the exploration of the immunocytochemical application of the developed monoclonal antibody against xanthohumol (XN). The intracellular fate of xanthohumol is an underexplored field in the research for the molecular mechanisms causing its wide range of effects in chemoprevention and gene expression involved in hepatic metabolism. The monoclonal antibody was used to elucidate possible targets for the binding of XN in a human mammary carcinoma cell line (MCF-7/6). Prior to the immunochemical experiments in growth medium with XN, its solubility and stability were investigated and found appropriate. They showed that the after 6 h of incubation at 37 °C, and even after 24 h, about 50% of the original amount of XN could not be retrieved in the

medium. The cellular uptake and distribution of XN in MCF-7/6 cells was investigated using an optimized immunocytochemistry technique. After incubation of MCF-7/6 cells, with 10 µM XN for 0.5 h up to 6 h, we observed primarily a granular nuclear staining, which intensified with increasing exposure times. Immunoprecipitation of cell lysates (treated with 10 µM XN for 2 h) revealed binding of xanthohumol to a fraction of proteins with a molecular weight below 20 kDa. All controls (blanks and negative) did not result in the same band pattern that was observed with the positive samples. Further analysis of the protein mixture via LC-MS/MS (Q-TOF) resulted in the identification of specific members of the histone family, *i.e.* histone H2A, H2B and H4. The identity of histone H2A was confirmed using immunodetection with a specific anti-histone H2A antibody.

# Samenvatting

Deze thesis beschrijft de ontwikkeling en validatie van twee analytische methodes voor de bepaling van fyto-oestrogenen in biologische matrices, meer bepaald een vloeistofchromatografische methode in combinatie met massaspectrometrische detectie (MS) (Atmosferische Druk Chemische Ionisatie) voor de bepaling van 13 fyto-oestrogenen (met inbegrip van intestinale metabolieten) in urine en serum en een niet-chromatografische methode gebruik makend van monoklonale antilichamen in een enzyme-gelinkte immunosorbent assay (ELISA) voor het hop-afgeleide xanthohumol (XN), isoxanthohumol (IX) en 8-prenylnaringenin (8-PN). Bovendien ligt de focus vooral op de toepassing van de ontwikkelde methodes als tools voor de analyse van echte stalen.

In Hoofdstuk 1 werden op basis van een literatuurstudie, de betreffende verbindingen, zijnde fyto-oestrogenen, besproken op verschillende niveaus zoals voedselbronnen, biologische activiteiten en beschikbaarheid en algemeen metabolisme. Fyto-oestrogenen worden gedefinieerd als plantaardige, niet-steroïdale secundaire metabolieten, die acties van endogene oestrogenen kunnen nabootsen en dit meestal door hun binding aan oestrogeen receptoren. De meerderheid van de fyto-oestrogenen zijn flavonoïden, gesubstitueerde fenolische verbindingen, die behoren tot drie groepen, *i.e.* isoflavonen (van soja (*Glycine max* (L.) Merr.), geprenyleerde flavonoïden (van hop (*Humulus lupulus* L.) en coumestanen (van rode klaver, alfalfa). De niet-flavonoïde lignanen (van lijnzaad (*Linum usitatissimum* L.) zijn een andere grote groep fyto-oestrogenen die ook dagelijks kunnen geconsumeerd worden via plantaardige voedingsmiddelen of via de inname van voedingssupplementen. Gelijktijdig staan fyto-oestrogenen vaak in de schijnwerpers als zeer interessante onderzoeksonderwerpen vanwege hun brede spectrum van activiteiten in de preventie en bescherming tegen bepaalde ziekten zoals kanker, hart- en vaatziekten en osteoporose. Hun gezondheidseigenschappen zijn grotendeels afhankelijk van hun biologische beschikbaarheid en bijgevolg dus de fysiologische levels die na consumptie bereikt worden. Uiteraard speelt het intestinale metabolisme ook een belangrijke rol bij de vele inter-individuele verschillen in niveaus van blootstelling aan hormonaal actieve verbindingen. Wanneer men de inname en de opname van fyto-oestrogenen en hun effecten op cellulair niveau wil bepalen, hetzij in een klinische omgeving of gecontroleerde

laboratoriumomstandigheden, zijn betrouwbare analytische methoden voor hun bepaling en kwantificering echter een eerste vereiste.

In Hoofdstuk 2 zijn de opbouw en de belangrijkste doelstellingen van deze thesis beschreven. Deze laatste waren onderdeel van een contractueel onderzoek naar fyto-oestrogenen uit voedingssupplementen. Meer bepaald hun biologische beschikbaarheid, fysiologische impact en toxische implicaties met betrekking tot de menselijke gezondheid (acron: FYTOES) ondersteund door de Federale Overheidsdienst (Gezondheid, Veiligheid van de Voedselketen en Leefmilieu).

Hoofdstuk 3 beschrijft de succesvolle ontwikkeling en optimalisatie van een nieuwe HPLC-APCI(MS) analysemethode voor de bepaling en de kwantificering van 13 fyto-oestrogenen, met inbegrip van hun precursoren en microbiële darmmetabolieten (genisteïne (GEN), daidzeïne (DAID), equol (EQ), dihydrodaidzeïne (DHD), *O*-desmethylangolensin (*O*-DMA), coumestrol (COUM), secoisolariciresinol (SECO), matairesinol (MAT), enterolacton (ENL), enterodiol (END), xanthohumol (XN), isoxanthohumol (IX) en 8-prenylnaringenine (8-PN)) in urine en serum. De eenvoudige staalvoorbereiding bestaat uit enzymatische deconjugatie gevolgd door vloeistof-vloeistof extractie (LLE) of vaste-fase-extractie (SPE) voor urine of serum, respectievelijk.

Gedurende de ontwikkeling van deze methode is allereerst de chromatografische scheiding van de analyten en interne standaard geoptimaliseerd, met behulp van omgekeerde fase chromatografie. Verschillende factoren zoals de stationaire fase, de samenstelling van de mobiele fase, pH, kolomtemperatuur en mobiele fase additieven zijn op punt gesteld om voor een adequate scheiding te zorgen in een korte run. De finale methode maakt gebruik van een XBridge C18-kolom bij een temperatuur van 55 °C met een mobiele fase-gradiënt bestaande uit water en een mengsel van methanol/acetonitrile (80:20, m/m), beide aangezuurd met 0.025% (v/v) mierenzuur. In 20 minuten worden de

verbindingen gescheiden met basislijn resolutie (behalve EQ, GEN en ENL; R < 1.5) en wordt de kolom geëquilibreerd voor een volgende run. Volledige resolutie werd bereikt door koppeling van het LC-gedeelte met een Agilent 1200 serie Multimode Source Detector. Vervolgens werden voor elke verbinding afzonderlijk, de interface en MS parameters geoptimaliseerd en de optimale ionisatie methode gekozen. Atmosferische druk chemische ionisatie (APCI), in de positieve mode, genereerde pseudomoleculaire ionen $[M + H]^+$ voor de meeste verbindingen, met uitzondering van SECO die $[M + H\text{-}2H_2O]^+$ als de hoofdpiek. Alleen END en ENL werden geïoniseerd via APCI in de negatieve modus ($[M - H]^-$), omdat dan hogere gevoeligheid werd verkregen. De fragmentorvoltage, die per molecule verschilt, werd ingesteld samen met de gekozen ionisatie methode. Alsook werden vier MSD signalen vastgelegd, met oog op de gevoeligheid en resolutie. Tenslotte werden de gainwaarden en MSD cyclustijden ingesteld per geprogrammeerd signaal.

Bovendien werd deze bio-analytische methode volledig gevalideerd (volgens de aanbevelingen van de FDA) op basis van selectiviteit, lineariteit, gevoeligheid, nauwkeurigheid en precisie. Bijgevolg bleek deze methode geschikt voor routinematige analyse van stalen met fyto-oestrogeen niveaus in het lagere ppb-bereik (LOD: urine: 0.2-7.7 ng/mL; serum: 1.4-20.4 ng/mL), met uitzondering van SECO (urine: 65.1 ng/mL; serum: 132.6 ng/mL), met lineariteit ($r^2 > 0.995$) voor alle analyten over de dynamische bereiken. Ook bleek een nauwkeurige en precieze kwantificering van de beoogde fyto-oestrogenen en hun metabolieten mogelijk met deze methode. De nauwkeurigheid (uitgedrukt als percentage relatieve fout; %RE) was binnen het acceptabele bereik van 20% en de inter-assay precisie voor urine en serum bedroeg niet meer dan 20%. De intra-assay precisie was minder dan 10% voor urine en serum voor alle analyten, behalve voor END en DAID en ENL in urine en serum, respectievelijk. Er werd ook een beoordeling van de matrixeffecten in urine en serum gemaakt naar richtlijnen van Matuszewski *et al.* [110]. Globaal werd er niet meer dan 15% variatie gevonden die toe te schrijven zou zijn aan matrixeffecten in urine en serum.

In Hoofdstuk 4 diende de ontwikkelde en gevalideerde LC/(APCI-)MS methode als een waardevolle tool in het analytische deel van een gerandomiseerde cross-over voedingstrial met co-supplementatie van soja-, hop-en/of vlas-gebaseerde voedingssupplementen door Bolca *et al.* [130]. Deze studie toont aan dat de biobeschikbaarheid van fyto-oestrogenen sterk bepaald wordt door het microbioom van elk individu en dat gepersonaliseerd screenings nuttig kunnen zijn vóór het gebruik van deze voedingssupplementen.

Hoofdstuk 5 geeft een overzicht van de ontwikkeling van monoklonale antistoffen tegen fyto-oestrogenen en hun latere toepassing in een enzyme-linked immunosorbent assay (ELISA) voor biologische matrices. Het doel was het ontwikkelen van monoklonale antistoffen tegen daidzeïne, genisteïne, coumestrol, xanthohumol (XN), isoxanthohumol (IX) en 8-prenylnaringenin (8-PN). Verschillende polyklonale antilichamen werden reeds ontwikkeld tegen fyto-oestrogenen zoals daidzeïne, genisteïne, equol, coumestrol en enterolacton, wat hun immunogeen vermogen bewijst. Monoklonale antilichamen, die de voordelen hebben van hun specificiteit, homogeniteit en de beschikbaarheid op lange termijn, zijn tot nu toe alleen beschikbaar tegen genisteïne, daidzeïne en equol. Niettemin, dient ermee rekening te worden gehouden, dat vóór de immunisatie, fyto-oestrogenen geconjugeerd moeten worden met bovien serum albumine (BSA), omdat met hun laag molecuulgewicht, ze normaal geen immuunrespons opwekken *in vivo*. Op die manier was de eerste stap in de ontwikkeling de koppeling van de verbindingen aan BSA, waarbij 4'-*O*-carboxymethylgenisteïne, -daidzeïne, -xanthohumol, -isoxanthohumol, -8-prenylnaringenine en 3-*O*-carboxymethylcoumestrol. Daarna werden BALB/c muizen geïmmuniseerd met de antigenen (d.w.z. fyto-oestrogeen-conjugaten), en door toepassing van een celfusie-procedure met polyethyleenglycol (PEG) op de muis miltcellen en myeloma NS0 cellen, werden de respectieve hybridoma's geconstrueerd. Met HAT selectiemedium, dat aminopterine bevat die de *de novo* pathway blokkeert, werden de gefuseerde B-cel-myeloma hybriden onderscheiden van de myeloom- en B-cellen. Dan, met een niet-competitieve screenings ELISA op basis van antigeen gecoate platen, konden enkel een paar hybridoma's worden geselecteerd, die antilichamen tegen XN, IX en 8-PN produceerden. Deze laatste antistoffen werden vervolgens ook getest in een indirecte, competitieve ELISA

om de affiniteit van de geproduceerde antilichamen voor de oorspronkelijke verbindingen te onderzoeken. De monoklonaliteit van de antilichamen werd vervolgens gewaarborgd via de methode van beperkende verdunning. De klonen van belang werden in cultuur gebracht in CELLine bioreactoren en grote hoeveelheden van monoklonale antilichamen konden elke week geoogst worden, dit gedurende enkele maanden. De monoklonaal antilichaam oplossingen werden verder opgezuiverd uit het medium gebruik makende van FPLC en HiTrap Proteïne G kolommen. In totaal werd 59 mg, 195 mg en 52 mg (gecontroleerd met behulp van gelelektroforese met Coomassie blauw kleuring) zuiver anti-XN, anti-IX en anti-8-PN verkregen.

Met behulp van de opgezuiverde monoklonale antilichamen, werden de verschillende stappen en parameters van de competitieve ELISA (verdunningen van de antigen coating, de blockingbuffer en het primaire en secundaire antilichaam; en het type van secundair antilichaam en enzymsubstraat, evenals de incubatietemperatuur en -tijd perioden) geoptimaliseerd. De definitieve ELISA-procedure maakt gebruik van immunogeen gecoate microtiterplaten en een peroxidase gelabeld antimuis-$IgG_1$ secundair antilichaam met ABTS als chromogeen substraat. Voor XN was de $IC_{50}$-waarde afgeleid van de standaard curve 62.91 ng/mL, en voor zowel IX en 8-PN 37.15 ng/mL. Via kruisreactiviteitsstudies werd aangetoond dat de monoklonale antilichamen zeer specifieke bindingscapaciteiten vertonen in een geoptimaliseerde competitive indirecte ELISA.

De ELISA-test is gevalideerd voor de kwantitatieve analyse van XN, IX en 8-PN in urine en serum. Een eenvoudige voorbehandelingsprocedure met behulp van een diëthylether extractie werd geoptimaliseerd, alsook werden de recovery's en matrixeffecten beoordeeld. De validiteit van de gevestigde assay werd getest en voor inter- en intra-assay variaties in urine werden waarden berekend van 2.32% en 1.91%, respectievelijk voor XN, 6.24% en 2.39%, respectievelijk voor IX en 7.18% en 0.74%, respectievelijk voor 8-PN. In serum bedroegen de gemiddelde inter- en intra-assay variaties 8.90% en 1.37%, respectievelijk voor XN, 6.13% en 1.57%, respectievelijk voor IX en 6.13% en 2.43%, respectievelijk voor 8-PN.

Bovendien, de methode vertoonde uitstekende nauwkeurigheid en significante correlatie met metingen door de gevestigde en gevalideerde HPLC-MS methode uit Hoofdstuk 3.

Hoofdstuk 6 handelt over de aanwending van een andere immunochemische toepassing van het monoklonale antilichaam tegen xanthohumol (XN). Het intracellulaire lot van xanthohumol is een nog weinig besproken deel in het onderzoek naar de moleculaire mechanismen van de brede waaier aan effecten in chemopreventie en genexpressie die betrokken zijn bij levermetabolisme. Het monoklonale antilichaam werd gebruikt voor het ophelderen van mogelijke doelwitten voor de binding van XN in een menselijke borstklier kanker cellijn (MCF-7/6). Voorafgaand aan de immunochemische experimenten in groeimedium met XN, zijn de oplosbaarheid en stabiliteit van XN onderzocht en aanvaardbaar bevonden. Deze experimenten toonden aan dat XN na 6 uur incubatie bij 37 °C, en zelfs na 24 u, ongeveer 50% van de oorspronkelijke hoeveelheid XN niet kon worden terug gevonden in het celmedium. De opname en distributie van XN in MCF-7/6 cellen werd onderzocht met behulp van een geoptimaliseerde immunocytochemische techniek. Na incubatie van MCF-7/6 cellen, met 10 µM XN voor 0.5 u tot 6 u, werd voornamelijk een granulaire nucleaire kleuring waargenomen, die versterkte met toenemende blootstellingstijden. Na immunoprecipitatie van cellysaten (behandeld met 10 µM XN voor 2 u) bleek dat XN binding gaf met een fractie van eiwitten met een moleculair gewicht kleiner dan 20 kDa. Alle controlestalen (blanco's en negatieve controles) leidden niet tot hetzelfde patroon dat werd waargenomen bij de positieve stalen. Verdere analyse van het eiwitmengsel via LC-MS/MS (Q-TOF) resulteerde in de identificatie van bepaalde leden van de histonfamilie, d.w.z. histon H2A, H2B en H4. De identiteit van histon H2A werd bevestigd met behulp van immunodetectie met een specifiek anti-histon H2A antilichaam.

# General discussion and future perspectives

The biological mechanisms of phytoestrogens and their potential application as health-beneficial component in food supplements, have been studied intensively over the last few decades. Unfortunately, the focus is hardly drawn on the renewal, extension and simplification of analytical methods for their determination, either qualitatively or quantitatively. Nevertheless, the basis of every complete decision-making in laboratory or clinical environment is based on accurate and precise detection tools and/or methods. Additionally, it is also a bonus if the methods are simple, do not require experienced staff or expensive equipment and offer a rapid solution in routine analysis of large numbers of samples.

The new or improved detection tools discussed in this thesis, as well as their applications, have been developed and run in view of the simplification of the analysis of phytoestrogens in biological matrices. Moreover, they can be a contribution to other research groups investigating pharmacokinetics of phytoestrogens, molecular biology and therapeutic applications with dietary supplements.

The developed LC/APCI-MS method for 13 phytoestrogens (including gut microbial metabolites) in urine and serum covers the simultaneous determination and quantification of the most important members of the 4 major groups of phytoestrogens, *i.e.* isoflavones, lignans, prenylated flavonoids and coumestans and includes also their most important biological metabolites. This analytical method has been applied to analyze the urinary and serum samples generated in a dietary intervention trial. For that type of trial, the LC-MS method proved its efficacy, moreover the possibility to determine all the targeted compounds in one run, with a minimal effort put in sample preparation and technical simplicity. Nevertheless, further improvement of this type of method, is warranted to maximize its contribution and value in clinical environments. More sophisticated techniques such as LC-MS/MS and UHPLC-MS/MS, which were not readily available during this research project, could significantly improve the speed and efficacy, without any loss of specificity and

accuracy. At the moment of finalization of this thesis and the generation of this chapter, literature search showed that some research groups successfully improved the sample preparation, method sensitivity and overall efficiency of the detection of some groups of phytoestrogens. The first [221] was conducted by applying an extraction-free method for the measurement of isoflavones and lignans with APPI, rather than using ESI or APCI. The latter [222] was realized by optimizing the speed through the use of UHPLC and improving sensitivity by using tandem MS technique. These improvements were applied for the detection of some but unfortunately not all of the compounds integrated in the method of this research project.

The establishment of the second and also very challenging part of this research project, was the development of a series of monoclonal antibodies against the most important members of the main groups in the class of phytoestrogens: genistein, daidzein, equol, enterodiol, enterolactone, xanthohumol, isoxanthohumol, 8-prenylnaringenin and coumestrol. Given their non-immunogenic nature, the synthesis of haptens of all these molecules and linking those to a protein was absolutely necessary, and successfully conducted for genistein, daidzein, coumestrol, isoxanthohumol, xanthohumol and 8-prenylnaringenin. Immunogens for the other compounds were not synthesized because of the limited time and lack of experienced staff. At the time the research project was conducted, only the generation of monoclonal antibodies against daidzein, genistein and equol were described by other research groups in literature. Therefore, the most innovative antibodies, were used for generation of a monoclonal based ELISA, immunocytochemistry and –precipitation. A number of aspects in the development, optimization and validation of an ELISA with monoclonal antibodies were explored during this project. Nevertheless, further investigation and exploration of the possibilities with the developed monoclonal antibodies would be useful to improve the sensitivity for measurement in biological samples. These antibodies, and especially the anti-xanthohumol antibody could serve as a very interesting tool for further exploration of the cellular target of this hop-derived compound. Xanthohumol has been suggested as an interesting natural compound with many health beneficial properties. The metabolism of xanthomol has already been thoroughly studied

through *in vitro* studies with human and rat liver microsomes, microorganisms and phase II enzymes. Also a few *in vivo* studies (in rats) [65, 210, 223] are available so far and very recently, Legette *et al.* [224] performed the first study to elucidate the pharmacokinetic parameters in humans. In the latter study experiments with a single oral dose of pure xanthohumol (20, 60 or 180 mg) revealed pharmacokinetic parameters showing a distinct biphasic absorption pattern with a first peak around 1 h and a second peak between 4-5 h after ingestion. Steady-state plasma concentrations were calculated to be 1.5, 18 and 40 µg/L for the 20, 60 and 180 mg doses, respectively. A half-life time of 20 h was observed for the lowest dose and 18 h for the highest dose. The biphasic pattern could be due to enterohepatic circulation, or absorption of redissolved xanthohumol that may have precipitated in intestinal fluids. Another important finding of that study is the parallel between human and animal pharmacokinetics. Because of the similarity of xanthohumol metabolism between animals and humans, findings can easily be translated to future clinical work. In conclusion this important *in vivo* human study revealed that the issue of poor bioavailability of xanthohumol, or plasma and tissue levels of xanthohumol, can be eliminated by using of a self-emulsifying isotropic mixture of xanthohumol, oleic acid, Tween 80 and propylene glycol for oral administration. This in turn opens up new perspectives for future cancer research on the efficacy of xanthohumol against a number of systemic tumors and enhances the importance of biological effects observed in cell culture studies. The tend to modify the transcription through demethylation and acetylation of histones in breast cancer cell lines has recently also been observed and described for the isoflavones genistein, daidzein and equol [225]. Therefore, especially the association of xanthohumol with histones and the possible influence on posttranslational modifications seems to be a highly interesting perspective in cancer research. The value of the developed anti-xanthohumol antibody in that field has already been expressed in literature [226].

# REFERENCES


1. Dixon, R.A., *Phytoestrogens.* Annual Review of Plant Biology, 2004. 55: p. 225-261.

2. Mazur, W. and Adlercreutz, H., *Naturally occurring oestrogens in food.* Pure and Applied Chemistry, 1998. 70(9): p. 1759-1776.

3. Bradbury, R.B. and White, D.E., *Estrogens and related substances in plants.* Vitam Horm, 1954. XII: p. 207-233.

4. Cos, P., De Bruyne, T., Apers, S., Vanden Berghe, D., Pieters, L. and Vlietinck, A.J., *Phytoestrogens: Recent developments.* Planta Medica, 2003. 69(7): p. 589-599.

5. Brzezinski, A. and Debi, A., *Phytoestrogens: the "natural" selective estrogen receptor modulators?* European Journal of Obstetrics Gynecology and Reproductive Biology, 1999. 85(1): p. 47-51.

6. Loewe, S., Lange, F. and Spohr, E., *Über weibliche Sexualhormone.* Biochem Zeitschr, 1927. 180: p. 1-26.

7. Farnsworth, N.R., Bingel, A.S., Cordell, G.A., Crane, F.A. and Fong, H.H.S., *Potential Value of Plants as Sources of New Antifertility Agents 2.* Journal of Pharmaceutical Sciences, 1975. 64(5): p. 717-754.

8. Axelson, M., Kirk, D.N., Farrant, R.D., Cooley, G., Lawson, A.M. and Setchell, K.D.R., *The Identification of the Weak Estrogen Equol [7-Hydroxy-3-(4'-Hydroxyphenyl)Chroman] in Human-Urine.* Biochemical Journal, 1982. 201(2): p. 353-357.

9. Adlercreutz, H., *Western Diet and Western Diseases - Some Hormonal and Biochemical-Mechanisms and Associations.* Scandinavian Journal of Clinical & Laboratory Investigation, 1990. 50: p. 3-23.

10. Rossouw, J.E., Anderson, G.L., Prentice, R.L., La Croix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A.A. and Howard, B.V., *Risks and benefits of estrogen plus progestin in healthy postmenopausal women - Principal results from the Women's Health Initiative randomized controlled trial.* Jama-Journal of the American Medical Association, 2002. 288(3): p. 321-333.

11. Banks, E., Beral, V., Bull, D., Reeves, G., Austoker, J., English, R., Patnick, J., Peto, R., Vessey, M. and Wallis, M., *Breast cancer and hormone-replacement therapy in the Million Women Study.* Lancet, 2003. 362(9382): p. 419-427.

12. Hu, J.Y. and Aizawa, T., *Quantitative structure-activity relationships for estrogen receptor binding affinity of phenolic chemicals.* Water Research, 2003. 37(6): p. 1213-1222.

13. Kurzer, M.S. and Xu, X., *Dietary phytoestrogens.* Annual Review of Nutrition, 1997. 17: p. 353-381.

14. Wang, H.J. and Murphy, P.A., *Isoflavone Composition of American and Japanese Soybeans in Iowa - Effects of Variety, Crop Year, and Location.* Journal of Agricultural and Food Chemistry, 1994. 42(8): p. 1674-1677.

15. Wang, H.J. and Murphy, P.A., *Isoflavone Content in Commercial Soybean Foods.* Journal of Agricultural and Food Chemistry, 1994. 42(8): p. 1666-1673.

16. Prasain, J.K., Carlson, S.H. and Wyss, J.M. *Flavonoids and age-related disease: Risk, benefits and critical windows.* Maturitas, 2010. 66(2): p. 163-171.

17. Atkinson, C., Frankenfeld, C.L. and Lampe, J.W., *Gut bacterial metabolism of the soy isoflavone daidzein: Exploring the relevance to human health.* Experimental Biology and Medicine, 2005. 230(3): p. 155-170.

18. Stevens, J.F., Taylor, A.W. and Deinzer, M.L., *Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography tandem mass spectrometry.* Journal of Chromatography A, 1999. 832(1-2): p. 97-107.

19. Nikolic, D., Li, Y., Chadwick, L.R., Pauli, G.F. and van Breemen, R.B., *Metabolism of xanthohumol and isoxanthohumol, prenylated flavonoids from hops (Humulus lupulus L.), by human liver microsomes.* Journal of Mass Spectrometry, 2005. 40(3): p. 289-299.

20. Possemiers, S., Bolca S., Grootaert, C., Heyerick, A., Decroos, K., Dhooge, W., De Keukeleire, D., Rabot, S., Verstraete, W. and Van de Wiele, T., *The prenylflavonoid isoxanthohumol from hops (Humulus lupulus L.) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine.* Journal of Nutrition, 2006. 136(7): p. 1862-1867.

21. Tekel', J., De Keukeleire, D., Rong, H., Daeseleire, E. and Van Peteghem, C., *Determination of the hop-derived phytoestrogen, 8-prenylnaringenin, in beer by gas chromatography/mass spectrometry.* Journal of Agricultural and Food Chemistry, 1999. 47(12): p. 5059-5063.

22. Mazur, W., *Phytoestrogen content in foods.* Baillieres Clinical Endocrinology and Metabolism, 1998. 12(4): p. 729-742.

23. Borriello, S.P., Setchell, K.D.R., Axelson, M. and Lawson, A.M., *Production and Metabolism of Lignans by the Human Fecal Flora.* Journal of Applied Bacteriology, 1985. 58(1): p. 37-43.

24. Heinonen, S., Nurmi, T., Liokkonen, K., Poutanen, K., Wähälä, K., Deyama, T.; Nishibe, S. and Adlercreutz, H., *In vitro metabolism of plant lignans: New precursors of mammalian lignans enterolactone and enterodiol.* Journal of Agricultural and Food Chemistry, 2001. 49(7): p. 3178-3186.

25. Franke, A.A., Custer, L.J., Cerna, C.M. and Narala, K., *Rapid HPLC Analysis of Dietary Phytoestrogens from Legumes and from Human Urine.* Proceedings of the Society for Experimental Biology and Medicine, 1995. 208(1): p. 18-26.

26. Setchell, K.D.R., Faughnan, M.S., Avades, T., Zimmer-Nechemias, L., Brown, N.M., Wolfe, B.E., Brashear, W.T., Desai, P., Oldfield, M.F., Botting, N.P. and Cassidy, A., *Comparing the pharmacokinetics of daidzein and genistein with the use of C-13-labeled tracers in premenopausal women.* American Journal of Clinical Nutrition, 2003. 77(2): p. 411-419.

27. Kuijsten, A., Arts, I.C., Vree, T.B. and Hollman, P.C., *Pharmacokinetics of enterolignans in healthy men and women consuming a single dose of secoisolariciresinol diglucoside.* Journal of Nutrition, 2005. 135(4): p. 795-801.

28. Nikolic, D., Li, Y., Chadwick, L.R. and van Breemen, R.B. , *In vitro studies of intestinal permeability and hepatic and intestinal metabolism of 8-prenylnaringenin, a potent phytoestrogen from hops (Humulus lupulus L.).* Pharmaceutical Research, 2006. 23(5): p. 864-872.

29. Rad, M., Hümpel, M., Schaefer, O., Schoemaker, R.C., Schleuning, W.D., Cohen, A.F. and Burggraaf, J., *Pharmacokinetics and systemic endocrine effects of the phyto-oestrogen 8-prenylnaringenin after single oral doses to postmenopausal women.* British Journal of Clinical Pharmacology, 2006. 62(3): p. 288-296.

30. Pang, Y., Nikolic, D., Zhu, D., Chadwick, L.R., Pauli, G.F., Farnsworth, N.R. and van Breemen, R.B., *Binding of the hop (Humulus lupulus L.) chalcone xanthohumol to cytosolic proteins in Caco-2 intestinal epithelliall cells.* Molecular Nutrition & Food Research, 2007. 51(7): p. 872-879.

31. Bolca, S., Possemiers, S., Maervoet, V., Huybrechts, .I, Heyerick, A., Vervarcke, S., Depypere, .H, De Keukeleire, D., Bracke, M., De Henauw, S., Verstraete, W. and Van de Wiele, T., *Microbial and dietary factors associated with the 8-prenylnaringenin producer phenotype: a dietary intervention trial with fifty healthy post-menopausal Caucasian women.* British Journal of Nutrition, 2007. 98(5): p. 950-959.

32. Day, A.J., DuPont, M.S., Ridley, S., Rhodes, M., Rhodes, M.J., Morgan, M.R. and Williamson, G., *Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity.* Febs Letters, 1998. 436(1): p. 71-75.

33. Hawksworth, G., Drasar, B.S. and Hill, M.J., *Intestinal Bacteria and Hydrolysis of Glycosidic Bonds.* Journal of Medical Microbiology, 1971. 4(4): p. 451-459.

34. Rowland, I., Faughnan, M., Hoey, L., Wähälä, K., Williamson, G. and Cassidy, A., *Bioavailability of phyto-oestrogens.* British Journal of Nutrition, 2003. 89: p. S45-S58.

35. Steensma, A., Noteborn, H.P., Jagt, R.C., Polman, T.H., Mengelers, M.J. and Kuiper, H.A., *Bioavailability of genistein, daidzein, and their glycosides in intestinal epithelial Caco-2 cells.* Environmental Toxicology and Pharmacology, 1999. 7(3): p. 209-211.

36. Setchell, K.D.R., Brown, N.M., Zimmer-Nechemias, L., Brashear, W.T., Wolfe, B.E., Kirschner, A.S. and Heubi, J.E., *Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability.* American Journal of Clinical Nutrition, 2002. 76(2): p. 447-453.

37. Zhang, Y., Hendrich, S. and Murphy, P.A., *Glucuronides are the main isoflavone metabolites in women.* Journal of Nutrition, 2003. 133(2): p. 399-404.

38. Adlercreutz, H., van der Wildt, J., Kinzel, J., Attalla, H., Wähälä, K., Mäkelä, T., Hase, T. and Fotsis, T., *Lignan and Isoflavonoid Conjugates in Human Urine.* Journal of Steroid Biochemistry and Molecular Biology, 1995. 52(1): p. 97-103.

39. Yilmazer, M., Stevens, J.F. and Buhler, D.R., *In vitro glucuronidation of xanthohumol, a flavonoid in hop and beer, by rat and human liver microsomes.* Febs Letters, 2001. 491(3): p. 252-256.

40. Schaefer, O., Bohlmann, R., Schleuning, W.D., Schulze-Forster, K. and Hümpel, M., *Development of a radioimmunoassay for the quantitative determination of 8-prenylnaringenin in biological matrices.* Journal of Agricultural and Food Chemistry, 2005. 53(8): p. 2881-2889.

41. Bolca, S., Urpi-Sarda, M., Blondeel, P., Roche, N., Vanhaecke, L., Possemiers, S., Al-Maharik, N., Botting, N., De Keukeleire, D., Bracke, M., Heyerick, A., Manach, C. and Depypere, H., *Disposition of soy isoflavones in normal human breast tissue.* American Journal of Clinical Nutrition, 2010. 91(4): p. 976-984.

42. Possemiers, S., Bolca, S., Eeckhaut, E., Depypere, H. and Verstraete, W., *Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community.* Fems Microbiology Ecology, 2007. 61(2): p. 372-383.

43. Setchell, K.D.R. and Cassidy, A., *Dietary isoflavones: Biological effects and relevance to human health.* Journal of Nutrition, 1999. 129(3): p. 758s-767s.

44. Kronenberg, F. and Hughes, C., *Exogenous and endogenous estrogens: An appreciation of biological complexity.* Menopause-the Journal of the North American Menopause Society, 1999. 6(1): p. 4-6.

45. Dechering, K., Boersma, C. and Mosselman, S., *Estrogen receptors alpha and beta: Two receptors of a kind?* Current Medicinal Chemistry, 2000. 7(5): p. 561-576.

46. Kuiper, G.G, Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S. and Gustafsson, J.A., *Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta.* Endocrinology, 1997. 138(3): p. 863-870.

47. Milligan, S.R., Kalita, J.C., Heyerick, A., Rong, H., De Cooman, L. and De Keukeleire, D., *Identification of a potent phytoestrogen in hops (Humulus lupulus L.) and beer.* Journal of Clinical Endocrinology & Metabolism, 1999. 84(6): p. 2249-2252.

48. Schaefer, O., Hümpel, M., Fritzemeier, K.H., Bohlmann, R. and Schleuning, W.D., *8-Prenylnaringenin is a potent ER alpha selective phytoestrogen present in hops and beer.* Journal of Steroid Biochemistry and Molecular Biology, 2003. 84(2-3): p. 359-360.

49. Humpel, M., Isaksson, P., Schaefer, O., Kaufmann, U., Ciana, P., Maggi, A. and Schleuning, W.D., *Tissue specificity of 8-prenylnaringenin: Protection from ovariectomy induced bone loss with minimal trophic effects on the uterus.* Journal of Steroid Biochemistry and Molecular Biology, 2005. 97(3): p. 299-305.

50. Heyerick, A., Vervarcke, S., Depypere, H., Bracke, M. and De Keukeleire, D., *A first prospective, randomized, double-blind, placebo-controlled study on the use of a standardized hop extract to alleviate menopausal discomforts.* Maturitas, 2006. 54(2): p. 164-175.

51. Mueller, S.O., Simon, S., Chae, K., Metzler, M. and Korach, K.S., *Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ER alpha) and ER beta in human cells.* Toxicological Sciences, 2004. 80: p. 14-25.

52. Possemiers, S., Heyerick, A., Robbens, V., De Keukeleire, D. and Verstraete, W., *Activation of proestrogens from hops (Humulus lupulus L.) by intestinal microbiota; Conversion of isoxanthohumol into 8-prenylnaringenin.* Journal of Agricultural and Food Chemistry, 2005. 53(16): p. 6281-6288.

53. Adlercreutz, C.H., Goldin, B.R., Gorbach, S.L., Höckerstedt, K.A., Watanabe, S., Hämäläinen, E.K., Markkanen, M.H., Mäkelä, T.H., Wähälä, K.T. and Adlercreutz T., *Soybean Phytoestrogen Intake and Cancer Risk.* Journal of Nutrition, 1995. 125(3): p. 757S-770S.

54. Sathyamoorthy, N., Wang, T.T.Y. and Phang, J.M., *Stimulation of Ps2 Expression by Diet-Derived Compounds.* Cancer Research, 1994. 54(4): p. 957-961.

55. Wang, T.T.Y., Sathyamoorthy, N. and Phang, J.M., *Molecular effects of genistein on estrogen receptor mediated pathways.* Carcinogenesis, 1996. 17(2): p. 271-275.

56. Gerhauser, C., Alt, A., Heiss, E., Gamal-Eldeen, A., Klimo, K., Knauft, J., Neumann, I., Scherf, H.R., Frank, N., Bartsch, H. and Becker, H., *Cancer chemopreventive activity of Xanthohumol, a natural product derived from hop.* Molecular Cancer Therapeutics, 2002. 1(11): p. 959-969.

57. Overk, C.R., Yao, P., Chadwick, L.R., Nikolic, D., Sun, Y., Cuendet, M.A., Deng, Y., Hedayat, A.S., Pauli, G.F., Farnsworth, N.R., van Breemen, R.B. and Bolton, J.L., *Comparison of the in vitro estrogenic activities of compounds from hops (Humulus lupulus) and red clover (Trifolium pratense).* Journal of Agricultural and Food Chemistry, 2005. 53(16): p. 6246-6253.

58. DeSantis, C., Siegel, R., Bandi, P. and Jemal, A., *Breast cancer statistics, 2011.* CA: A Cancer Journal for Clinicians, 2011. 61(6): p. 409-418.

59. Adlercreutz, H., Heinonen, S.M. and Penalvo-Garcia, J., *Phytoestrogens, cancer and coronary heart disease.* Biofactors, 2004. 22(1-4): p. 229-236.

60. Messina, M.J., Persky, V., Setchell, K.D. and Barnes, S., *Soy Intake and Cancer Risk - a Review of the in-Vitro and in-Vivo Data.* Nutrition and Cancer-an International Journal, 1994. 21(2): p. 113-131.

61. Adlercreutz, H., Mousavi, Y., Clark, J., Höckerstedt, K., Hämäläinen, E., Wähälä, K., Mäkelä, T. and Hase, T., *Dietary Phytoestrogens and Cancer - In vitro and In vivo Studies.* Journal of Steroid Biochemistry and Molecular Biology, 1992. 41(3-8): p. 331-337.

62. Ingram, D., Sanders, K., Kolybaba, M. and Lopez, D., *Case-control study of phyto-oestrogens and breast cancer.* Lancet, 1997. 350(9083): p. 990-994.

63. Zava, D.T. and Duwe, G., *Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro.* Nutrition and Cancer-an International Journal, 1997. 27(1): p. 31-40.

64. Wang, C.F. and Kurzer, M.S., *Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells.* Nutrition and Cancer-an International Journal, 1997. 28(3): p. 236-247.

65. Stevens, J.F. and Page, J.E., *Xanthohumol and related prenylflavonoids from hops and beer: to your good health!* Phytochemistry, 2004. 65(10): p. 1317-1330.

66. Miranda, C.L., Yang, Y.-H., Henderson, M.C., Stevens, J.F., Santana-Rios, G., Deinzer, M.L. and Buhler, D.R., *Prenylflavonoids from hops inhibit the metabolic activation of the carcinogenic heterocyclic amine 2-amino-3-methylimidazo[4,5-F] quinoline, mediated by cDNA-expressed human CYP1A2.* Drug Metabolism and Disposition, 2000. 28(11): p. 1297-1302.

67. Henderson, M.C., Miranda, C.L., Stevens, J.F., Deinzer, M.L. and Buhler, D.R., *In vitro inhibition of human P450 enzymes by prenylated flavonoids from hops, Humulus lupulus.* Xenobiotica, 2000. 30(3): p. 235-251.

68. Dietz, B.M., Kang, Y.-H., Liu, G., Eggler, A.L., Yao, P., Chadwick, L.R., Pauli, G.F., Farnsworth, N.R., Mesecar, A.D., van Breemen, R.B. and Bolton, J.L., *Xanthohumol isolated from Humulus lupulus inhibits menadione-induced DNA damage through induction of quinone reductase.* Chemical Research in Toxicology, 2005. 18(8): p. 1296-1305.

69. Miranda, C.L., Aponso, G.L.M., Stevens, J.F., Deinzer, M.L. and Buhler, D.R. *Prenylated chalcones and flavanones as inducers of quinone reductase in mouse Hepa 1c1c7 cells.* Cancer Letters, 2000. 149(1-2): p. 21-29.

70. Vanhoecke, B., Lust, S., Janssens, A., Philippé, J., Bracke, M. and Offner, F., *Xanthohumol induces apoptosis in B-CLL mediated by caspase 9 following downregulation of bcL-xL and mcl-1.* Blood, 2005. 106(11): p. 339b-339b.

71. Zhao, F., Nozawa, H., Daikonnya, A., Kondo, K. and Kitanaka, S., *Inhibitors of nitric oxide production from hops (Humulus lupulus L.).* Biological & Pharmaceutical Bulletin, 2003. 26(1): p. 61-65.

72. Rong, H.J., Boterberg, T., Maubacj, J., Stove, C., Depypere, H., Van Slambrouck, S., Seereyn, R., De Keukeleire, D., Mareel, M., Bracke, M., *8-Prenylnaringenin, the phytoestrogen in hops and beer, upregulates the function of the E-cadherin/catenin complex in human mammary carcinoma cells.* European Journal of Cell Biology, 2001. 80(9): p. 580-585.

73. Brunelli, E., Pinton, G., Chianale, F., Graziani, A., Appendino, G. and Moro, L., *8-Prenylnaringenin inhibits epidermal growth factor-induced MCF-7 breast cancer cell proliferation by targeting phosphatidylinositol-3-OH kinase activity.* Journal of Steroid Biochemistry and Molecular Biology, 2009. 113(3-5): p. 163-170.

74. Brunelli, E., Minassi, A., Appendino, G. and Moro, L., *8-Prenylnaringenin, inhibits estrogen receptor-alpha mediated cell growth and induces apoptosis in MCF-7 breast cancer cells.* Journal of Steroid Biochemistry and Molecular Biology, 2007. 107(3-5): p. 140-148.

75. Monteiro, R., Becker, H., Azevedo, I. and Calhau, C., *Effect of hop (Humulus lupulus L.) flavonoids on aromatase (estrogen synthase) activity.* Journal of Agricultural and Food Chemistry, 2006. 54(8): p. 2938-2943.

76. Monteiro, R., Faria, A., Azevedo, I. and Calhau, C., *Modulation of breast cancer cell survival by aromatase inhibiting hop (Humulus lupulus L.) flavonoids.* Journal of Steroid Biochemistry and Molecular Biology, 2007. 105(1-5): p. 124-130.

77. Pepper, M.S., Hazel, S.J., Hümpel, M. and Schleuning, W.-D., *8-prenylnaringenin, a novel phytoestrogen, inhibits angiogenesis in vitro and in vivo.* Journal of Cellular Physiology, 2004. 199(1): p. 98-107.

78. Bolca, S., Li, J., Nikolic, D., Roche, N., Blondeel, P., Possemiers, S., De Keukeleire, D., Bracke, M., Heyerick, A., van Breemen, R.B. and Depypere, H., *Disposition of hop prenylflavonoids in human breast tissue.* Molecular Nutrition & Food Research, 2010. 54: p. S284-S294.

79. Anderson, J.W., Johnstone, B.M. and Cooknewell, M.E., *Meta-analysis of the Effects of Soy Protein-Intake on Serum-Lipids.* New England Journal of Medicine, 1995. 333(5): p. 276-282.

80. Tikkanen, M.J. and Adlercreutz, H., *Dietary soy-derived isoflavone phytoestrogens - Could they have a role in coronary heart disease prevention?* Biochemical Pharmacology, 2000. 60(1): p. 1-5.

81. Greaves, K.A., Parks, J.S., Williams, J.K. and Wagner, J.D., *Intact dietary soy protein, but not adding an isoflavone-rich soy extract to casein, improves plasma lipids in ovariectomized cynomolgus monkeys.* Journal of Nutrition, 1999. 129(8): p. 1585-1592.

82. Crouse, J.R., Morgan, T., Terry, J.G., Ellis, J., Vitolins, M. and Burke, G.L., *A randomized trial comparing the effect of casein with that of soy protein containing varying amounts of isoflavones on plasma concentrations of lipids and lipoproteins.* Archives of Internal Medicine, 1999. 159(17): p. 2070-2076.

83. Hodgson, J.M., Puddey, I.B., Beilin, L.J., Mori, T.A. and Croft, K.D., *Supplementation with isoflavonoid phytoestrogens does not alter serum lipid concentrations: A randomized controlled trial in humans.* Journal of Nutrition, 1998. 128(4): p. 728-732.

84. Lissin, L.W. and Cooke, J.P., *Phytoestrogens and cardiovascular health.* Journal of the American College of Cardiology, 2000. 35(6): p. 1403-1410.

85. Tham, D.M., Gardner, C.D. and Haskell, W.L., *Clinical review 97 - Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence.* Journal of Clinical Endocrinology & Metabolism, 1998. 83(7): p. 2223-2235.

86. Gooderham, M.J., Adlercreutz, H., Ojala, S.T., Wähälä, K. and Holub, B.J., *A soy protein isolate rich in genistein and daidzein and its effects on plasma isoflavone concentrations, platelet aggregation, blood lipids and fatty acid composition of plasma phospholipid in normal men.* Journal of Nutrition, 1996. 126(8): p. 2000-2006.

87. van der Schouw, Y.T., Kreijkamp-Kaspers, S., Peeters, P.H., Keinan-Boker, L., Rimm, E.B. and Grobbee, D.E., *Prospective study on usual dietary phytoestrogen intake and cardiovascular disease risk in Western women.* Circulation, 2005. 111(4): p. 465-471.

88. Kilkkinen, A., Erlund, I., Virtanen, M.J., Alfthan, G., Ariniemi, K. and Virtamo, J., *Serum enterolactone concentration and the risk of coronary heart disease in a case-cohort study of Finnish male smokers.* American Journal of Epidemiology, 2006. 163(8): p. 687-693.

89. Vanharanta, M.P.K., Vanharanta, M. P. K., Voutilainen, S., Lakka, T. A., van der Lee, M., Adlercreutz, H. and Salonen, J. T., *Reduced risk of acute coronary events at high levels of a mammalian lignan, enterolactone: A prospective population based case-control study.* Circulation, 1999. 100(18): p. 870-870.

90. de Kleijn, M.J.J., van der Schouw, Y.T., Wilson, P.W., Grobbee, D.E. and Jacques, P.F., *Dietary intake of phytoestrogens is associated with a favorable metabolic cardiovascular risk profile in postmenopausal US women: The Framingham Study.* Journal of Nutrition, 2002. 132(2): p. 276-282.

91. van der Schouw, Y.T., Pijpe, A., Lebrun, C.E., Bots, M.L., Peeters, P.H., van Staveren, W.A., Lamberts, S.W. and Grobbee D.E., *Higher usual dietary intake of phytoestrogens is associated with lower aortic stiffness in postmenopausal women.* Arteriosclerosis Thrombosis and Vascular Biology, 2002. 22(8): p. 1316-1322.

92. Peterson, J., Dwyer, J., Adlercreutz, H., Scalbert, A., Jacques, P. and McCullough M.L. , *Dietary lignans: physiology and potential for cardiovascular disease risk reduction.* Nutrition Reviews, 2010. 68(10): p. 571-603.

93. Miranda, C.L., Stevens, J.F., Ivanov, V., McCall, M., Frei, B., Deinzer, M.L. and Buhler, D.R., *Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavanones in vitro.* Journal of Agricultural and Food Chemistry, 2000. 48(9): p. 3876-3884.

94. Stevens, J.F., Miranda, C.L., Frei, B. and Buhler, D.R., *Inhibition of peroxynitrite-mediated LDL oxidation by prenylated flavonoids: The alpha,beta-unsaturated keto functionality of 2'-hydroxychalcones as a novel antioxidant pharmacophore.* Chemical Research in Toxicology, 2003. 16(10): p. 1277-1286.

95. Tabata, N., Ito, M., Tomoda, H. and Ömura, S., *Xanthohumols, diacylglycerol acyltransferase inhibitors, from Humulus lupulus.* Phytochemistry, 1997. 46(4): p. 683-687.

96. Wang, C.C., Prasain, J.K. and Barnes, S., *Review of the methods used in the determination of phytoestrogens.* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2002. 777(1-2): p. 3-28.

97. Committee on Toxicity of Chemicals in Food, Consumer products and the Environment, *Phytoestrogens and health.* Food Standards Agency, 2003: p. 22-93.

98. Wilkinson, A.P., Wähälä, K. and Williamson, G., *Identification and quantification of polyphenol phytoestrogens in foods and human biological fluids.* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2002. 777(1-2): p. 93-109.

99. Wu, Q.L., Wang, M.F. and Simon, J.E., *Analytical methods to determine phytoestrogenic compounds.* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2004. 812(1-2): p. 325-355.

100. Setchell, K.D.R., Welsh, M.B. and Lim, C.K., *High-Performance Liquid-Chromatographic Analysis of Phytoestrogens in Soy Protein Preparations with Ultraviolet, Electrochemical and Thermospray Mass-Spectrometric Detection.* Journal of Chromatography, 1987. 386: p. 315-323.

101. Rybak, M.E., Parker, D.L. and Pfeiffer, C.M., *Determination of urinary phytoestrogens by HPLC-MS/MS: A comparison of atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI).* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2008. 861(1): p. 145-150.

102. King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C. and Olah, T., *Mechanistic investigation of ionization suppression in electrospray ionization.* Journal of the American Society for Mass Spectrometry, 2000. 11(11): p. 942-950.

103. Matuszewski, B.K., Constanzer, M.L. and Chavez-Eng, C.M., *Matrix effect in quantitative LC/MS/MS analyses of biological fluids: A method for determination of finasteride in human plasma at picogram per milliliter concentrations.* Analytical Chemistry, 1998. 70(5): p. 882-889.

104. Mei, H., Hsieh, Y.S., Nardo, C., Xu, X.Y., Wang, S.Y., Ng, K. and Korfmacher, W.A. , *Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery.* Rapid Communications in Mass Spectrometry, 2003. 17(1): p. 97-103.

105. Souverain, S., Rudaz, S. and Veuthey, J.L., *Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures.* Journal of Chromatography A, 2004. 1058(1-2): p. 61-66.

106. Choi, B.K., Hercules, D.M. and Gusev, A.I., *Effect of liquid chromatography separation of complex matrices on liquid chromatography-tandem mass spectrometry signal suppression.* Journal of Chromatography A, 2001. 907(1-2): p. 337-342.

107. Gomez, M.J., Petrovic, M., Fernandez-Alba, A.R. and Barcelo, D., *Determination of pharmaceuticals of various therapeutic classes by solid-phase extraction and liquid chromatography-tandem mass spectrometry analysis in hospital effluent wastewaters.* Journal of Chromatography A, 2006. 1114(2): p. 224-233.

108. Kloepfer, A., Quintana, J.B. and Reemtsma, T., *Operational options to reduce matrix effects in liquid chromatography-electrospray ionization-mass spectrometry analysis of aqueous environmental samples.* Journal of Chromatography A, 2005. 1067(1-2): p. 153-160.

109. Kang, J., Hick, L.A. and Price, W.E., *Using calibration approaches to compensate for remaining matrix effects in quantitative liquid chromatography/electrospray ionization multistage mass spectrometric analysis of phytoestrogens in aqueous environmental samples.* Rapid Communications in Mass Spectrometry, 2007. 21(24): p. 4065-4072.

110. Matuszewski, B.K., Constanzer, M.L. and Chavez-Eng, C.M., *Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS.* Analytical Chemistry, 2003. 75(13): p. 3019-3030.

111. Koivunen, M.E. and Krogsrud, R.L., *Principles of immunochemical techniques used in clinical laboratories.* Labmedicine, 2006. 37(8): p. 490-497.

112. Harlow, E. and Lane, D., *Antibodies - a laboratory manual.*, 1988, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

113. *Antibody structure and classification*. Molecular Probes. Available from: www.invitrogen.com. (accessed on 10th September 2012)

114. Catty, D., *Antibodies Volume I - a practical approach*. Practical Approach Series, ed. D. Rickwood and B.D. Hames. 1988, Oxford: IRL Press.

115. *Technical guide for ELISA*. Available from: www.kpl.com. (accessed on 10th September 2012)

116. Coons, A.H., Creech, H.J. and Joons, R.N., *Immunological properties of an antibody containing a fluorescent group.* Proc Soc Exp Biol Med, 1941. 47: p. 200-202.

117. Mao, S., Lorette, C. and Kent, U.M., *Overview of antibody use in immunocytochemistry.*, in *Methods in Molecular Biology*, L.C. Javois, Editor 1999, Humana Press Inc: Totowa, NJ. p. 3-10.

118. Bonifacino, J.S., Dell'Angelica, E.C. and Springer, T.A., *Immunoprecipitation.* Current Protocols in Molecular Biology, 2001. 48: p. 10.16.1-10.16.29.

119. Cimino, C.O., Shelnutt, S.R., Ronis, M.J.J., Badger, T.M., An LC-MS method to determine concentrations of isoflavones and their sulfate and glucuronide conjugates in urine. Clinica Chimica Acta, 1999. 287: p. 69-82.

120. Franke, A.A. Custer, L.J., Wilkens, L.R., Le Marchand, L.L., Nomura, A.M., Goodman, M.T., Kolonel, L.N., Liquid chromatographic-photodioide array mass spectrometric analysis of dietary phytoestrogens from human urine and blood. Journal of Chromatography B, 2002. 777: p. 45-69.

121. Valentin-Blasini, L., Blount, B.C., Rogers, H.S. and Needham, L.L., *HPLC-MS/MS method for the measurement of seven phytoestrogens in human serum and urine.* Journal of Exposure Analysis and Environmental Epidemiology, 2000. 10(6): p. 799-807.

122. Grace, P.B., Mistry, N.S., Carter, M.H., Leathem, A.J.C. and Teale, P., *High throughput quantification of phytoestrogens in human urine and serum using liquid chromatography/tandem mass spectrometry (LC-MS/MS).* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2007. 853(1-2): p. 138-146.

123. Grace, P.B., Taylor, J.I., Botting, N.P., Fryatt, T., Oldfield, M.F. and Bingham, S.A., *Quantification of isoflavones and lignans in urine using gas chromatography/mass spectrometry.* Analytical Biochemistry, 2003. 315(1): p. 114-121.

124. *Agilent Multimode Ion Source*. Available from: www.agilent.com. (accessed on 24th September 2012).

125. Bolca, S., Possemiers, S., Herregat, A., Huybrechts, I., Heyerick, A., De Vriese, S., Verbruggen, M., Depypere, H., Bracke, M., De Henauw, S., Verstraete, W. and Van de Wiele T., *Microbial and dietary factors are associated with the equol producer phenotype in healthy postmenopausal women.* Journal of Nutrition, 2007. 137(10): p. 2242-2246.

126. *Guidance for Industry, Bioanalytical method validation.* US Department of Health and Human Services Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER) 2001.

127. Bansal, S. and DeStefano, A., *Key elements of bioanalytical method validation for small molecules.* Aaps Journal, 2007. 9(1): p. E109-E114.

128. Bambagiotti-alberti, M., Coran, S.A., Ghiara, C. and Giannellini, V., *Revealing the Mammalian Lignan Precursor Seciosolariciresinol Diglucoside in Flax Seed by Ionspray Mass-Spectrometry.* Rapid Communications in Mass Spectrometry, 1994. 8(8): p. 595-598.

129. Schmidt, T.J., Hemmati, S., Fuss, E. and Alfermann, A.W., *A combined HPLC-UV and HPLC-MS method for the identification of lignans and its application to the lignans of Linum usitatissimum L. and L. bienne Mill.* Phytochemical Analysis, 2006. 17(5): p. 299-311.

130. Bolca, S., Wyns, C., Depypere, H., De Keukeleire, D., Bracke, M., Verstraete, W. and Heyerick, A., *Cosupplementation of Isoflavones, Prenylflavonoids, and Lignans Alters Human Exposure to Phytoestrogen-Derived 17 beta-Estradiol Equivalents.* Journal of Nutrition, 2009. 139(12): p. 2293-2300.

131. Rice, S., Mason, H.D. and Whitehead, S.A., *Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa-luteal cells.* Journal of Steroid Biochemistry and Molecular Biology, 2006. 101: p. 216-225.

132. Power, K.A. and Thompson, L.U., *Can the combination of flaxseed and its lignans with soy and its isoflavones reduce the growth stimulatory effect of soy and its isoflavones on established breast cancer?* Molecular Nutrition and Food Research, 2007. 51: p. 845-856.

133. van Meeuwen, J.A., van der Berg, M., Sanderson, J.T., Verhoef, A. and Piersma, A.H., *Estrogenic effects of mixtures of phyto- and synthetic chemicals on uterine growth of prepubertal rats.* Toxicology Letters, 2007. 170: p. 165-176.

134. Bowey, E., Adlercreutz, H. and Rowland, I.R. *Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats.* Food and Chemical Toxicology, 2003. 41: p. 631-636.

135. Possemiers, S., Rabot, S., Espin J.C., Bruneau, A., Philippe, C., González-Sarrias, A., Heyerick, A., Tomás-Barberán, F.A., De Keukeleire, D. and Verstraete, W., *Eubacterium limosum activates isoxanthohumol form hops ( Humulus lupulus L.) into the potent phytoestrogen 8-prenylnaringenin in vitro and in rat intestine.* Journal of Nutrition, 2008. 138: p. 1310-1316.

136. Setchell, K.D.R., Brown, N.M. and Lydeking-Olsen, E., *The clinical importance of the metabolite equol - a clue to the effectiveness of soy and its isoflavones.* Journal of Nutrition, 2002. 132: p. 3577-3584.

137. Possemiers, S., Bolca, S., Grootaert, C., Heyerick, A., Decroos, K., Dhooge, W., De Keukeleire, D., Rabot, S., Verstraete, W. and Van de Wiele T., *The prenylflavonoid isoxanthohumol from hops ( Humulus lupulus L.) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine.* Journal of Nutrition, 2006. 136: p. 1862-1867.

138. Setchell, K.D.R., Lawson, A.M., Borriello, S.P., Harkness, R., Gordon, H., Morgan, D.M.L., Kirk, D.N., Adlercreutz, H., Anderson, L.C. and Axelson, M., *Lignan Formation in Man - Microbial Involvement and Possible Roles in Relation to Cancer.* Lancet, 1981. 2(8236): p. 4-7.

139. Kostelac, D., Rechkemmer, G. and Briviba, K., *Phytoestrogens modulate binding response of estrogen receptors à and á to the estrogen response element.* Journal of Agricultural and Food Chemistry, 2003. 51: p. 7632-7635.

140. Milligan, S.R., Kalita, J.C., Heyerick, A., Rong, H., De Cooman, L. and De Keukeleire, D., *Identification of a potent phytoestrogen in hops (Humulus lupulus L.) and beer.* Journal of Clinical Endocrinology and Metabolism, 1999. 83(6): p. 2249-2252.

141. Mueller, S.O., Simon, S., Chae, K., Metzler, M. and Korach, K.S., *Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor α (ERα) and ERβ in human cells.* Toxicological Sciences, 2004. 80: p. 14-25.

142. Gu, L., House, S.E., Prior, R.L., Fang, N., Ronis, M.J.J., Clarkson, T.B., Wilson, M.E. and Badger, T.M., *Metabolic phenotype of isoflavones differ among female rats, pigs, monkeys, and women.* Journal of Nutrition, 2006. 136: p. 1215-1221.

143. Possemiers, S., Bolca, S., Eeckhaut, E., Depypere, H. and Verstraete, W., *Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community.* Fems Microbiology Ecology, 2007. 61: p. 372-383.

144. Possemiers, S., Heyerick, A., Robbens, V., De Keukeleire, D. and Verstraete, W., *Activation of pro-estrogens from hops (Humulus lupulus L.) by intestinal microbiota; conversion of isoxanthohumol into 8-prenylnaringenin.* Journal of Agricultural and Food Chemistry, 2005. 53: p. 6281-6288.

145. Peeters, P.H.M., Slimani, N., van der Schouw, Y.T., Grace, P.B., Navarro, C., Tjonneland, A., Olsen, A., Clavel-Chapelon, F., Touillaud, J., Boutron-Ruault, M. -C., Jenab, M., Kaaks, R., Linseisen, J., Trichopoulou, A., Trichopoulos, D., Dilis, V., Boeing, H., Weikert, C., Overvad, K., Pala, V., Palli, D., Panico, S., Tumino, R., Vineis, P., Bueno-de-Mesquita, H.B., van Gils, C.H., Skeie, G., Jakszyn, P., Hallmans, G., Berglund, G., Key, T.J., Travis, R., Riboli, E. and Bingham, S.A., *Variations in plasma phytoestrogen concentrations in European adults.* Journal of Nutrition, 2007. 137: p. 1294-1300.

146. Bolca, S., Possemiers, S., Herregat, A., Huybrechts, I., Heyerick, A., De Vriese, S., Verbruggen, M., Depypere, H., De Keukeleire, D., Bracke, M., De Henauw, S., Verstraete, W. and Van de Wiele, T., *Microbial and dietary factors are associated with the equol producer phenotype in healthy postmenopausal women.* Journal of Nutrition, 2007. 137: p. 2242-2246.

147. Kuijsten, A., Arts, I.C.W., van 't Veer, P. and Hollman, P.C.H., *The relative bioavailability of enterolignans in humans is enhanced by milling and crushing of flaxseed.* Journal of Nutrition, 2005. 135: p. 2812-2816.

148. Mathey, J., Lamothe, V., Coxam, V., Potier, M., Sauvant, P. and Bennetau-Pelissero, C., *Concentrations of isoflavones in plasma and urine of post-menopausal women chronically ingesting high quantities of soy isoflavones.* Journal of Pharmaceutical and Biomedical Analysis, 2006. 41(3): p. 957-965.

149. Gardner, C.D., Chatterjee, L.M. and Franke, A.A., *Effects of isoflavone supplements vs. soy foods on blood concentrations of genistein and daidzein in adults.* Journal of Nutritional Biochemistry, 2009. 20(3): p. 227-234.

150. Wyns, C., Bolca, S., De Keukeleire, D. and Heyerick, A., *Development of a high-throughput LC/APCI-MS method for the determination of thirteen phytoestrogens including gut microbial metabolites in human urine and serum.* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2010. 878(13-14): p. 949-956.

151. Hall, M.C., O'Brien, B. and McCormack, T., *Equol producer status, salivary estradiol profile and urinary excretion of isoflavones in Irish Caucasian women, following ingestion of soymilk.* Steroids, 2007. 72: p. 64-70.

152. Bartels, H. and Bohmer, M., *Eine Mikromethode zur Kreatininebestimmung.* Clinica Chimica Acta, 1971. 31: p. 81-85.

153. Junge, W., Wilke, B., Halabi, A. and Klein, G., *Determination of reference intervals for serum creatinine, creatinine excretion and creatinine clearance with an enzymatic and a modified Jaffe method.* Clinica Chimica Acta, 2004. 344(1-2): p. 137-148.

154. Bland, J.M. and Altman, D.G., *Statistical methods for assessing agreement between two methods of clinical measurement.* Lancet, 1986. 1(8476): p. 307-310.

155. Bratcher, T.L., Moran, M.A. and Zimmer, W.J., *Tables of sample sizes in the analysis of variance.* Journal of Quality Technology, 1970. 2: p. 156-164.

156. Fritsche, S. and Steinhart, H., *Occurence of hormonally active compounds in food: a review.* European Food Research and Technology, 1999. 209: p. 153-179.

157. Clavel, T., Henderson, G., Alpert, C.-A., Philippe, C., Rigottier-Gois, L., Doré, J. and Blaut, M., *Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans.* Applied and Environmental Microbiology, 2005. 71(10): p. 6077-6085.

158. Wang, L.Q., Meselhy, M.R., Li, Y., Qin, G.W. and Hattori, M., *Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone.* Chemical & Pharmaceutical Bulletin, 2000. 48(11): p. 1606-1610.

159. Clavel, T., Henderson, G., Engst, W., Doré, J. and Blaut, M., *Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside.* Fems Microbiology Ecology, 2006. 55(3): p. 471-478.

160. Decroos, K., Vanhemmens, S., Cattoir, S., Boon, N. and Verstraete, W., *Isolation and characterization of an equol producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions.* Archives of Microbiology, 2004. 183: p. 45-55.

161. Yokoyama, S. and Suzuki, T., *Isolation and characterization of a novel equol-producing bacterium from human feces.* Bioscience Biotechnology and Biochemistry, 2008. 72(10): p. 2660-2666.

162. Maruo, T., Sakamoto, M., Ito, C., Toda, T. and Benno, Y., *Adlercreutzia equolifaciens gen. nov., sp nov., an equol-producing bacterium isolated from human faeces, and emended description of the genus Eggerthella* International Journal of Systematic and Evolutionary Microbiology, 2008. 58: p. 1221-1227.

163. Calloway, D.H., Colasito, D.J. and Mathews, R.D., *Gases produced by human intestinal microflora.* Nature, 1966. 212: p. 1238-1239.

164. Gibson, G.R., Cummings, J.H. and Macfarlane, G.T., *Competition for hydrogen between sulphate-reducing bacteria and methanogenic bacteria from the human large intestina.* Journal of Applied Bacteriology, 1988. 65: p. 241-247.

165. Bolca, S., Possemiers, S., Maervoet, V., Huybrechts, I., Heyerick, A., Vervarcke, S., Depypere, H., De Keukeleire, D., Bracke, M., De Henauw, S., Verstraete, W. and Van de Wiele, T., *Microbial and dietary factors associated with the 8-prenylnaringenin producer phenotype: a dietary intervention trial with fifty healthy post-menopausal Caucasian women.* British Journal of Nutrition, 2007. 98(5): p. 950-959.

166. Nesbitt, P.D., Lam, Y. and Thompson, L.U., *Human metabolism of mammalian lignan precursors in raw and processed flaxseed.* American Journal of Clinical Nutrition, 1999. 69: p. 549-555.

167. Rad, M., Hümpel, M., Schoemaker, R.C., Schleuning, W.-D., Cohen, A.F. and Burggraaf, J., *Pharmacokinetics and systemic endocrine effects of the phyto-oestrogen 8-prenylnaringenin after single oral doses to postmenopausal women.* British Journal of Clinical Pharmacology, 2006. 62: p. 288-296

168. Kortenkamp, A., *Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals.* Environmental Health Perspectives, 2007. 115 (Suppl. 1): p. 98-105.

169. Rowland, I.R., Faughnan, M., Hoey, L., Wähälä, K., Williamson, G. and Cassidy, A., *Bioavailability of phyto-estrogens.* British Journal of Nutrition, 2003. 89: p. S45-S58.

170. Rong, H., Zhao, Y., Lazou, K., De Keukeleire, D., Milligan, S.R. and Sandra, P., *Quantitation of 8-prenylnaringenin, a novel phytoestrogen in hops (Humulus lupulus L.), hop products, and beers, by benchtop HPLC-MS using electrospray ionization.* Chromatographia, 2000. 51(9-10): p. 545-552.

171. Magalhaes, P.J., Guido, L.F., Cruz, J.M. and Barros, A.A., *Analysis of xanthohumol and isoxanthohumol in different hop products by liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry.* Journal of Chromatography A, 2007. 1150(1-2): p. 295-301.

172. Prasain, J.K., Arabshahi, A., Moore, D.R., Greendale, G.A., Wyss, J.M. and Barnes, S., *Simultaneous determination of 11 phytoestrogens in human serum using a 2 min liquid chromatography/tandem mass spectrometry method.* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2010. 878(13-14): p. 994-1002.

173. Bauminger, S., Lindner, H.R., Perel, E. and Arnon, R., *Antibodies to a Phytoestrogen - Antigenicity of Genistein Coupled to a Synthetic Polypeptide.* Israel Journal of Medical Sciences, 1969. 44: p. 567-578.

174. Lapcik, O., Stursa, J., Kleinova, T., Vitkova, M., Dvoráková, H., Klijdus, B. and Moravcová, J., *Synthesis of hapten and conjugates of coumestrol and development of immunoassay.* Steroids, 2003. 68(14): p. 1147-1155.

175. Adlercreutz, H., Wang, G.J., Lapcik, O., Hampl, R., Wähälä, K., Mäkelä, T., Lusa, K., Talme, M. and Mikola, H., *Time-resolved fluoroimmunoassay for plasma enterolactone.* Analytical Biochemistry, 1998. 265(2): p. 208-215.

176. Bennetau-Pelissero, C., Le Houérou, C., Lamothe, V., Le Menn, F., Babin, P. and Bennetau, B., *Synthesis of haptens and conjugates for ELISAs of phytoestrogens. Development of the immunological tests.* Journal of Agricultural and Food Chemistry, 2000. 48(2): p. 305-311.

177. Lapcik, O., Hampl, R., Al-Maharik, N., Salakka, A., Wähälä, K. and Adlercreutz, H., *A novel radioimmunoassay for daidzein.* Steroids, 1997. 62(3): p. 315-320.

178. Lapcik, O., Hampl, R., Hill, M., Wähälä, K., Al-Maharik, N. and Adlercreutz, H., *Radioimmunoassay of free genistein in human serum.* Journal of Steroid Biochemistry and Molecular Biology, 1998. 64(5-6): p. 261-268.

179. Lapcik, O., Vitková, M., Klejdus, B., Al-Maharik, N. and Adlercreutz, H., *Immunoassay for biochanin A.* Journal of Immunological Methods, 2004. 294(1-2): p. 155-163.

180. Kohen, F., Lichter, S., Gayer, B., De Boever, J. and Lu, L.J.W., *The measurement of the isoflavone daidzein by time resolved fluorescent immunoassay: a method for assessment of dietary soya exposure.* Journal of Steroid Biochemistry and Molecular Biology, 1998. 64(3-4): p. 217-222.

181. Kohen, F., Gayer, B., Amir-Zaltsman, Y., Ben-Hur, H., Thomas, E. and Lu, L.J., *A nonisotopic enzyme-based immunoassay for assessing human exposure to genistein.* Nutrition and Cancer-an International Journal, 1999. 35(1): p. 96-103.

182. Talbot, D.C.S., Ogborne, R.M., Dadd, T., Adlercreutz, H., Barnard, G., Bugel, S., Kohen, F., Marlin, S., Piron, J., Cassidy, A. and Powell, J., *Monoclonal antibody-based time-resolved fluorescence immunoassays for daidzein, genistein, and equol in blood and urine: Application to the isoheart intervention study.* Clinical Chemistry, 2007. 53(4): p. 748-756.

183. Al-Maharik, N.I., Katlia, S.A. and Wähälä, K., *Regioselective mono-O-carboxymethylation of polyhdroxyisoflavones.* Molecules Online 1999. 3: p. 20-24.

184. Gester, S., Metz, P., Zierau, O. and Vollmer, G., *An efficient synthesis of the potent phytoestrogens 8-prenylnaringenin and 6-(1,1-dimethylallyl)naringenin by europium(III)-catalyzed Claisen rearrangement.* Tetrahedron, 2001. 57(6): p. 1015-1018.

185. Hosoda, H., Sakai, Y., Yoshida, H., Miyairi, S., Ishii, K., and Nambara, T., *Studies on Steroids. Preparation of Steroid N-Hydroxysuccinimide Esters and Their Reactivities with Bovine Serum-Albumin.* Chemical & Pharmaceutical Bulletin, 1979. 27(3): p. 742-746.

186. Yatsimirskaya, E.A., Gavrilova, E.M., Egorov, A.M. and Levashov, A.V., *Preparation of Conjugates of Progesterone with Bovine Serum-Albumin in the Reversed Micellar Medium.* Steroids, 1993. 58(11): p. 547-550.

187. Brown, G. and Ling, N.R., *Murine monoclonal antibodies.*, in *Antibodies Volume I - a practical approach*, D. Catty, Editor 1988, IRL Press: Oxford. p. 81-104.

188. Harlow, E. and Lane, D., *Electrophoresis*, in *Using Antibodies: A Laboratory Manual*, J. Cuddihy, T. Kuhlmann, and P. Barker, Editors. 1988, CSHL Press. p. 424-426.

189. Shah, V.P., Midha, K.K., Findlay, J.W., Hill, H.M., Hulse, J.D., McGilveray, I.J., McKay, G., Miller, K.J., Patnaik, R.N., Powell, M.L., Tonelli, A., Viswanathan, C.T. and Yacobi A., *Bioanalytical method validation--a revisit with a decade of progress.* Pharmaceutical Research, 2000. 17(12): p. 1551-1557.

190. Findlay, J.W.A., Smith, W.C., Lee, J.W., Nordblom, G.D., Das, I., DeSilva, B.S., Khan, M.N. and Bowsher, R.R., *Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective.* Journal of Pharmaceutical and Biomedical Analysis, 2000. 21(6): p. 1249-1273.

191. Power, F.B., Tutin, F. and Rogerson, H., *The constituents of hops.* J. Chem. Soc., 1913. 103: p. 1267-1292.

192. Verzele, M., Stockx, J., Fontijn, F. and Anteunis, M., *Xanthohumol, a New Natural Chalkone.* Bulletin Des Societes Chimiques Belges, 1957. 66(1): p. 452-475.

193. Arimoto-Kobayashi, S., Sugiyama, C., Harada, N., Takeuchi, M., Takemura, M. and Hayatsu, H., *Inhibitory effects of beer and other alcoholic beverages on mutagenesis and DNA adduct formation induced by several carcinogens.* Journal of Agricultural and Food Chemistry, 1999. 47(1): p. 221-230.

194. Miranda, C.L., Stevens, J.F., Helmrich, A., Henderson, M.C., Rodriguez, R.J., Yang, Y.H., Deinzer, M.L., Barnes, D.W. and Buhler D.R., *Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (Humulus lupulus) in human cancer cell lines.* Food and Chemical Toxicology, 1999. 37(4): p. 271-285.

195. Vanhoecke, B., Derycke, L., Van Marck, V., Depypere, H., De Keukeleire, D. and Bracke, M., *Antiinvasive effect of xanthohumol, a prenylated chalcone present in hops (Humulus lupulus L.) and beer.* International Journal of Cancer, 2005. 117(6): p. 889-895.

196. Jacob, C., Jamier, V. and Ba, L.A., *Redox active secondary metabolites.* Current Opinion in Chemical Biology, 2011. 15(1): p. 149-155.

197. Yang, J.Y., Della-Fera, M.A., Rayalam, S. and Baile, C.A., *Effect of xanthohumol and isoxanthohumol on 3T3-L1 cell apoptosis and adipogenesis.* Apoptosis, 2007. 12(11): p. 1953-1963.

198. Strathmann, J., Klimo, K., Sauer, S.W., Okun, J.G., Prehn, J.H. and Gerhäuser, C., *Xanthohumol-induced transient superoxide anion radical formation triggers cancer cells into apoptosis via a mitochondria-mediated mechanism.* Faseb Journal, 2010. 24(8): p. 2938-2950.

199. Buckwold, V.E., Wilson, R.J., Nalca, A., Beer, B.B., Voss, T.G., Turpin, J.A., Buckheit, R.W., Wei, J., Wenzel-Mathers, M., Walton, E.M., Smith, R.J., Pallansch, M., Ward, P., Wells, J., Chuvala, L., Sloane, S., Paulman, R., Russell, J., Hartman, T. and Ptak, R., *Antiviral activity of hop constituents against a series of DNA and RNA viruses.* Antiviral Research, 2004. 61(1): p. 57-62.

200. Mizobuchi, S. and Sato, Y., *A New Flavanone with Antifungal Activity Isolated from Hops.* Agricultural and Biological Chemistry, 1984. 48(11): p. 2771-2775.

201. Wang, Q., Ding, Z.H., Liu, J.K. and Zheng, Y.T., *Xanthohumol, a novel anti-HIV-1 agent purified from Hops Humulus lupulus.* Antiviral Research, 2004. 64(3): p. 189-194.

202. Bhattacharya, S., Virani, S., Zavro, M. and Haas, G.J., *Inhibition of Streptococcus mutans and other oral streptococci by hop (Humulus lupulus L.) constituents.* Economic Botany, 2003. 57(1): p. 118-125.

203. Jeong, H.M., Han, E.H., Jin, Y.H., Choi, Y.H., Lee, K.Y. and Jeong, H.G., *Xanthohumol from the hop plant stimulates osteoblast differentiation by RUNX2 activation.* Biochemical and Biophysical Research Communications, 2011. 409(1): p. 82-89.

204. Nozawa, H., *Xanthohumol, the chalcone from beer hops (Humulus lupulus L.), is the ligand for farnesoid X receptor and ameliorates lipid and glucose metabolism in KK-A(y) mice.* Biochemical and Biophysical Research Communications, 2005. 336(3): p. 754-761.

205. Casaschi, A., Maiyoh, G.K., Rubio, B.K., Li, R.W., Adeli, K. and Theriault, A.G., *The chalcone xanthohumol inhibits triglyceride and apolipoprotein B secretion in HepG2 cells.* Journal of Nutrition, 2004. 134(6): p. 1340-1346.

206. Lee, I.S., Lim, J., Gal, J., Kang, J.C., Kim, H.J., Kang, B.Y. and Choi, H.J., *Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells.* Neurochemistry International, 2011. 58(2): p. 153-160.

207. Monteiro, R., Calhau, C., Silva, A.O., Pinheiro-Silva, S., Guerreiro, S., Gärtner, F., Azevedo, I. and Soares, R., *Xanthohumol inhibits inflammatory factor production and angiogenesis in breast cancer xenografts.* Journal of Cellular Biochemistry, 2008. 104(5): p. 1699-1707.

208. Dorn, C., Kraus, B., Motyl, M., Weiss, T.S., Gehrig, M., Schölmerich, J., Heilmann, J. and Hellerbrand, C., *Xanthohumol, a chalcon derived from hops, inhibits hepatic inflammation and fibrosis.* Molecular Nutrition & Food Research, 2010. 54: p. S205-S213.

209. Gao, X.H., Deeb, D., Liu, Y., Dulchavsky, S.A. and Gautam, S.C., *Immunomodulatory activity of xanthohumol: inhibition of T cell proliferation, cell-mediated cytotoxicity and Th1 cytokine production through suppression of NF-kappa B.* Immunopharmacology and Immunotoxicology, 2009. 31(3): p. 477-484.

210. Avula, B., Ganzera, M., Warnick, J.E., Feltenstein, M.W., Sufka, K.J. and Khan, I.A., *High-performance liquid chromatographic determination of xanthohumol in rat plasma, urine, and fecal samples.* Journal of Chromatographic Science, 2004. 42(7): p. 378-382.

211. Braune, A., Loh, G., Sczesny, S., Blaut, M. and Braune, A. , *Recovery and metabolism of xanthohumol in germ-free and human microbiota-associated rats.* Molecular Nutrition & Food Research, 2010. 54(10): p. 1405-1413.

212. Yilmazer, M., Stevens, J.F., Deinzer, M.L. and Buhler, D.R. , *In vitro biotransformation of xanthohumol, a flavonoid from hops (Humulus lupulus), by rat liver microsomes.* Drug Metabolism and Disposition, 2001. 29(3): p. 223-231.

213. Ruefer, C.E., Gerhäuser, C., Frank, N., Becker, H. and Kulling, S.E., *In vitro phase II metabolism of xanthohumol by human UDP-glucuronosyltransferases and sulfotransferases.* Molecular Nutrition & Food Research, 2005. 49(9): p. 851-856.

214. Wyns, C., Derycke, L., Soenen, B., Bolca, S., Deforce, D., Bracke, M. and Heyerick A., *Production of monoclonal antibodies against hop-derived (Humulus lupulus L.) prenylflavonoids and the development of immunoassays.* Talanta, 2011. 85(1): p. 197-205.

215. Derycke, L., Stove, C., Vercoutter-Edouart, A.S., De Wever, O., Dollé, L., Colpaert, N., Depypere, H., Michalski, J.C. and Bracke, M., *The role of non-muscle myosin IIA in aggregation and invasion of human MCF-7 breast cancer cells.* International Journal of Developmental Biology, 2011. 55(7-9): p. 835-840.

216. Van Steendam, K., Tilleman, K., De Ceuleneer, M., De Keyser, F., Elewaut, D. and Deforce D., *Citrullinated vimentin as an important antigen in immune complexes from synovial fluid of rheumatoid arthritis patients with antibodies against citrullinated proteins.* Arthritis Research & Therapy, 2010. 12(4): R132.

217. Stevens, J.F., Taylor, A.W., Clawson, J.E. and Deinzer, M.L., *Fate of xanthohumol and related prenylflavonoids from hops to beer.* Journal of Agricultural and Food Chemistry, 1999. 47(6): p. 2421-2428.

218. Motyl, M., Kraus, B. and Heilmann, J., *Uptake of xanthohumol in human liver and intestinal cells.* Planta Medica, 2009. 75(9): p. 977-977.

219. Harikumar, K.B., Kunnumakkara, A.B., Ahn, K.S., Anand, P., Krishnan, S., Guha, S. and Aggarwal, B.B., *Modification of the cysteine residues in I kappa B alpha kinase and NF-kappa B (p65) by xanthohumol leads to suppression of NF-kappa B-regulated gene products and potentiation of apoptosis in leukemia cells.* Blood, 2009. 113(9): p. 2003-2013.

220. Link, A., Balaguer, F. and Goel, A., *Cancer chemoprevention by dietary polyphenols: Promising role for epigenetics.* Biochemical Pharmacology, 2010. 80(12): p. 1771-1792.

221. Parker, D.L., Rybak, M.E. and Pfeiffer, C.M., *Phytoestrogen biomonitoring: an extractionless LC-MS/MS method for measuring urinary isoflavones and lignans by use of atmospheric pressure photoionization (APPI).* Analytical and Bioanalytical Chemistry, 2012. 402: p. 1123–1136.

222. Yuan, Y., Qiu, X., Nikolic, D., Dahl, J.H. and van Breemen, R.B., *Method development and validation for UHPLC-MS-MS determination of hop prenylflavonoids in human serum.* Journal of AOAC International, 2012. 95(6): p. 1744–1749.

223. Legette, L., Ma, L., Reed, R.L., Miranda, C.L., Christensen, J.M., Rodriguez-Proteau, R., Stevens, J.F., *Pharmacokinetics of xanthohumol and metabolites in rats after oral and intravenouw adminsitration.* Molecular Nutrition and Food Research, 2012. 56(3): p. 466-474.

224. Legette L., Karnpracha, C., Reed, R.L., Choi, J., Bobe, G., Christensen, J.M., Rodriguez-Proteau, R., Purnell, J.Q. and Stevens, J.F., *Human pharmacokinetics of xanthohumol, an antihyperglycemic flavonoid from hops.* Molecular and Nutritional Food Research, 2014. 58: p. 248-255.

225. Dagdemir, A., Durif, J., Ngollo, M., Bignon, Y.-J. and Bernard-Gallon, D., *Histone lysine trimethylation or acetylation can be modulated by phytoestrogen, estrogen or anti-HDAC in breast cancer cell lines.* Epigenomics, 2013. 5(1): p. 51–63.

226. Kapoor, S., *Xanthohumol and it's emerging anti-neoplastic effects: Beyond it's role in breast carcinomas.* Molecular and Nutritional Food Research, 2012. 56: 1760.

# APPENDIX

**Table A.1:** **Summary of LC/MS and LC/MS-MS methods for the simultaneous detection of phytoestrogens (isoflavones and lignans) in biological samples.**

| Chromatographic methods | | | | |
|---|---|---|---|---|
| Method | Analytes | Matrix | Sensitivity | Ref. |
| HPLC/(APCI)MS | DAID, DHD, GEN, DHG, *O*-DMA, EQ | urine | 5 ng/mL (EQ: 200 ng/mL) | [119] |
| HPLC/(ESI)MS | DAID, DHD, GEN, DHG, GLYC, *O*-DMA, END, ENL & 3 others | urine/plasma | LOQ urine: 1–39 nM LOQ plasma: 1–29 nM | [120] |
| HPLC/(APCI)MS-MS | DAID, GEN, EQ, *O*-DMA, MAT, END, ENL, COUM | urine/serum | urine: 0.2–9.3 ng/mL serum: 0.1–2.4 ng/mL | [121] |
| HPLC/(ES)MS-MS | DAID, GEN, GLYC, EQ, *O*-DMA, END, ENL | urine | 10 pg/mL (EQ: 100 pg/mL) | [123] |
| HPLC/(ES)MS-MS | DAID, GEN, EQ, *O*-DMA, END, ENL | urine | 0.05–0.4 ng/mL | [101] |
| HPLC/(APCI)MS-MS | DAID, GEN, EQ, *O*-DMA, END, ENL | urine | 0.05–2.7 ng/mL | [101] |

Abbreviations: APCI: atmospheric pressure chemical ionization, ESI: electrospray ionization, MS(-MS): (tandem) mass spectrometry, Mw: molecular weight, DAID: daidzein (Mw: 254 g/mol), DHD: dihydrodaidzein (Mw: 256 g/mol), GEN: genistein (Mw: 270 g/mol), DHG: dihydrogenistein (Mw: 272 g/mol), GLYC: glycitein (Mw: 284 g/mol), EQ: equol (Mw: 242 g/mol), *O*-DMA: *O*-desmethylangolensin (Mw: 258 g/mol), MAT: matairesinol (Mw: 358 g/mol), END: enterodiol (Mw: 302 g/mol), ENL: enterolactone (Mw: 298 g/mol), COUM: coumestrol (Mw: 268 g/mol).

**Table A.2:** **Summary of immunoassays for the detection of phytoestrogens (isoflavones and lignans) in biological samples.**

| Immunoassays | | | | |
|---|---|---|---|---|
| Method | Compounds | Type Ab | Sensitivity | Ref. |
| RIA | 8-PN | polyclonal | 0.3 ng/mL | [40] |
| RIA | COUM | polyclonal | 12 pg/tube | [172] |
| TR-FIA | ENL | polyclonal | 1.5–540 nmol/L | [173] |
| ELISA | C7 serie: DAID, GEN, BIOCHANIN A, EQ<br>C8 serie: DAID, FORM | polyclonal | $IC_{50}$ : 10 ng/mL<br>(DAID: 0.8 ng/mL)<br>$IC_{50}$: 15.6, 7.8 ng/mL | [174] |
| RIA | DAID | polyclonal | 0.4 pg/tube | [175] |
| TR-FIA | GEN | polyclonal | 1.2 – 2.8 pg/tube | [176] |
| RIA | BIOCHANIN A | polyclonal | 3.1 pg/tube | [177] |
| ELISA | BIOCHANIN A | polyclonal | 5.3 pg/well | [177] |
| TR-FIA | DAID | monoclonal | 0.5 ng/mL | [178] |
| ELISA | GEN | monoclonal | 0.5 ng/well | [179] |
| TR-FIA | DAID, GEN, EQ | monoclonal | DAID: 3.9 nmol/L<br>GEN: 8.7–88.8 nmol/L<br>(serum – urine)<br>EQ: 2.2 nmol/L | [180] |

Abbreviations: ELISA: enzyme-linked immunosorbent assay, RIA: radioimmunoassay, TR-FIA: time-resolved fluoroimmunoassay, $IC_{50}$: concentration of competitor that gives 50% inhibition, Mw: molecular weight, DAID: daidzein (Mw: 254 g/mol), GEN: genistein (Mw: 270 g/mol), ENL: enterolactone (Mw: 298 g/mol), COUM: coumestrol (Mw: 268 g/mol), FORM: formononetin (Mw: 268 g/mol), EQ: equol (Mw: 242 g/mol), BIOCHANIN A (Mw: 284 g/mol).

# CURRICULUM VITAE

## Personalia


Name: Ciska Wyns

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Date of birth: April 1st 1983

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## Education

1995 - 2001: Secondary school,

Latin - Mathematics, Sint-Pietersinstituut, Oostkamp (1995 – 1999);
Latin - Mathematics, Sint-Jozefshumaniora, Brugge (1999 – 2001)

2001 – 2006: Master in Pharmaceutical Sciences, Ghent University, Gent.

Graduated with great distinction

Master thesis: *Study of the bioavailability of phytoestrogens.* Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmaceutical Sciences, Ghent University. Promoter: Prof. Dr. D. De Keukeleire.

2008 Course 'Advanced Academic English: writing skills' (2008), UCT, Ghent University.

Graduated

## Professional experience

2006 – 2012: PhD student, Laboratory of Phytochemistry and Pharmacognosy, Faculty of Pharmaceutical sciences, Ghent University, Gent.

December 2012 – present: Head of R&D, KeyPharm laboratories, Oostkamp.

## A1 publications:

- **Wyns, C.**, Bolca, S., De Keukeleire, D. and Heyerick, A. *Development of a high-throughput LC/APCI-MS method for the determination of thirteen phytoestrogens including gut microbial metabolites in human urine and serum.* Journal of Chromatography B, 2010. 878(13-14): p. 949-956.

- Bolca, S., **Wyns, C.**, Possemiers, S., Depypere, H., De Keukeleire, D., Bracke, M., Verstraete, W. and Heyerick A. *Cosupplementation of isoflavones, prenylflavonoids, and lignans alters human exposure to phytoestrogen-derived 17β-estradiol equivalents.* Journal of Nutrition, 2009. 139(12): p. 2293-300.

- **Wyns, C.,** Derycke, L., Soenen, B., Bolca, S., Deforce, D., Bracke, M. and Heyerick A. *Production of monoclonal antibodies against hop-derived (Humulus lupulus L.) prenylflavonoids and the development of immunoassays.* Talanta, 2011. 85(1): p. 197-205.

- **Wyns, C.**, Van Steendam, K., Vanhoecke, B., Deforce, D., Bracke, M. and Heyerick A. *Prenylated chalcone xanthohumol associates with histones in breast cancer cells – a novel target identified by a monoclonal antibody.* Molecular Nutrition and Food Research, 2012. 56(11): p. 1688-1696.

## C1 publications:

- **Wyns, C.**, Bolca, S., De Keukeleire, D. and Heyerick, A. *Development of a high-throughput LC-APCI-MS method for the determination of 13 phytoestrogens (including gut microbial metabolites) in human urine and serum.* Planta Medica, 2008. 74: p. 1100.

## Attended conferences and seminars:

- Annual BSMS Meeting on mass Spectrometry, Leuven, Belgium, February 2007. Passive participation.

- Waters Seminar: Efficient HPLC and UPLC method development and validation, Ghent, Belgium, March 2007. Passive participation.

- BSMS meeting, Beerse, Belgium, February 2008. Passive participation.

- Waters seminar: Pharmaceutical Method development and validation, Ghent, Belgium, April 2008. Passive participation.

- 7th Joint Meeting of AFERP, GA, ASF, PSE & SIF: Natural Products with Pharmaceutical, Nutraceutical, Cosmetic and Agrochemical Interest, Athens, Greece, August 2008. Poster.

- 2nd ISHS International 'Humulus Symposium', Ghent, Belgium, September 2008. Posters (2).

- NVMS – BSMS International Conference on Mass Spectrometry, Kerkrade, The Netherlands, March 2009. Poster presentation.

- Scientific Afternoon at the Faculty of Pharmaceutical Sciences, Ghent, Belgium, May 2009. Lecture and posters (2).

- NVMS-BSMS 4th International Conference on Polyphenols and Health, Harrogate, UK, December 2009. Poster.

*'Omnia aliena sunt, tempus tantum nostrum est' – Seneca*