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DETERMINATION AND IDENTIFICATION OF POLYPHENOLS AND THEIR METABOLITES IN BIOLOGICAL MATRICES

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ACADEMIC DISSERTATION

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ABSTRACT

Isoflavonoid phytoestrogens are secondary plant metabolites, which structurally or functionally resemble 17β-estradiol and they originally received attention due to breeding problems affecting ewes grazing on subterranean clover. Later research of phytoestrogens has been focusing on the possible beneficial effects as oestrogen agonists or anti-estrogens.

Due to their health promoting effects, the knowledge of the occurrence of isoflavonoids and their metabolites in food and biological fluids as well as the better understanding of their metabolic pathways have been the main aspects in the research field.

The literature review introduces the biological significance of isoflavonoids in plants along with various analytical techniques used for the determination of these compounds in biological matrices. This is followed by a discussion of the isoflavone metabolism in humans, rodents, and ruminants.

The experimental part focuses on the synthetic methods used for the preparation of the isoflavonoids, and on four studies in which they were used. In the first two studies cow milk was analysed. It was shown that commercial organic cow milk contains high levels of equol along with much lower levels of the other isoflavonoids typically found in milk and milk products. The levels of equol detected in organic milk were significantly higher than the levels found in normal milk which corresponds to the fodder that the cows are fed. In the following study five Finnish Ayrshire cows were subjected to a red clover rich diet in order to study the metabolism of the isoflavones futher. Equol and a hitherto unquantifed metabolite, 3',7-dihydroxyisoflavan was detected and quantitatively measured in milk samples.

In another study, the metabolisation of genistein fatty acid ester was studied after (oral or parenteral) administration to adult female rhesus monkeys. It turned that levels of genistein fatty acid ester levels depended of the form of administration, and it may be possible to introduce intact genistein ester molecules into plasma by parenteral, but not oral administration.

The last study, focuses on the metabolism of the soy isoflavones daidzein, genistein, and glycitein in humans. After oral administration of these isoflavones through ingestion of soy enriched food, daily urine samples of the volunteers were analysed. This led to identification of several metabolites and to the proposal of the metabolic pathways of the isoflavones ingested.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Hoikkala, Antti; Mustonen, Eeva; Saastamoinen, Ilkka; Jokela, Tuija; Taponen, Juhani; Saloniemi, Hannu; Wahala, Kristiina: High levels of equol in organic skimmed Finnish cow milk, Molecular Nutrition & Food Research (2007), 51(7), 782-786.

II Hoikkala Antti, Kontio Lea, Mustonen Eeva, Saastamoinen Ilkka, Jokela Tuija, Taponen Juhani, Halmemies Anni, Vanhatalo Aila and Wähälä Kristiina: Red clover derived isoflavone metabolites in bovine milk, Manuscript, 2016.

III Badeau, Maija; Tikkanen, Matti J.; Appt, Susan E.; Adlercreutz, Herman; Clarkson, Thomas B.; Hoikkala, Antti; Wähälä, Kristiina; Mikkola, Tomi S. : Determination of plasma genistein fatty acid esters following administration of genistein or genistein 4'7-O-dioleate in monkeys, Biochimica et Biophysica Acta, Molecular and Cell Biology of Lipids (2005), 1738(1-3), 115-120.

IV Heinonen, Satu-Maarit; Hoikkala, Antti; Wahala, Kristiina; Adlercreutz, Herman: Metabolism of the soy isoflavones daidzein, genistein and glycitein in human subjects. Identification of new metabolites having an intact isoflavonoid skeleton, Journal of Steroid Biochemistry and Molecular Biology (2004), 87(4-5), 285-299.

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

$AcO^{(-)}$	acetate
APCI	atmospheric pressure chemical ionisation
BA	biochanin A
BSA	bovine serum albumin
BSTFA	*N,O*-bis-(trimethylsilyl)-trifluoroacetamide
CE	capillary electrophoresis
COU	coumestrol
DA	Daidzein
DAD	diode array detector
DART	direct analysis in real-time
DEAE	diethyl-aminoethyl
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
END	enterodiol
ENL	entrolactone
ESI	electrospray ionisation
EtOH	ethanol
EQ	equol
FIA	fluoroimmunoassay
FO	formononetin
GC	gas chromatography
GEN	genistein
GLY	glycitein
HCl	hydrochloric acid
HMDS	hexamethyldisilazane
HN	heated nebulizer
HPLC	high performance liquid chromatography
ID	isotope dilution
LDL	low density lipoprotein
MALDI	matrix-assisted laser desorption ionisation
MeOH	methanol
MS	mass spectrometry
MTBE	methyl *tert*-butyl ether
O-DMA	*O*-demethylangolensin
PR	prunetin
RIA	radioimmunoassay
SIM	selected ion monitoring
SIR	single ion recording
SPE	solid phase extraction
TBDMS	*t*-butyldimethylsilyl
TEAP	triethylaminohydroxypropyl

TMS	trimethylsilyl
TMSCl	trimethylsilyl chloride
TR-FIA	time-resolved fluoroimmunoassay
UHPLC	ultra high performance liquid chromatography
UV	ultra violet
QAE	quaternary aminoethyl

1 INTRODUCTION

Polyphenols were originally referred as “vegetable tannins” due to the use of plant extracts in the conversion of animal hide into leather. Originally, the term plant polyphenol was reserved to “water-soluble phenolic compounds having molecular weights between 500 and 3000 Daltons and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatine and other proteins from solution” [Swain, 1962]. Since this definition basically included just the so-called condensed tannins, hydrolysable tannins and phlorotannins, Quideau et al. [Quideau et al. 2011] recently proposed a wider definition of polyphenols to include also other compounds such as flavonoids, which encompass 8000 classified structures [Andersen O.M., 2006]. According to Quideau, “the term polyphenol should be used to define plant secondary metabolites derived exclusively from shikimate-derived phenylpropanoid and/or the polyketide pathway(s) featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression”.

Isoflavonoid phytoestrogens are secondary plant metabolites. They are structurally or functionally similar to 17β-estradiol and originally received attention due to breeding problems affecting ewes grazing on subterranean clover [Bennetts et al., 1946]. Later research of phytoestrogens has been focusing on the possible beneficial effects as oestrogen agonists or anti-estrogens. Their possible effects have been implicated in the etiology of hormone-dependent cancers [Adlercreutz, 2002; Magee and Rowland, 2004], cardiovascular disease [Anthony et al., 1996; Dang, 2005], osteoporosis [Dang, 2005], menopausal symptoms among others [Kuhnle et al., 2008; Stark, 2002].

The work in this thesis concentrates on isoflavonoid phytoestrogens and their metabolites. The majority of the publications in the phytoestrogen field have been focused on the studies of the intact compounds present in plants. Relatively little, however, is known about the fate of these compounds when ingested. Furthermore, since metabolites have been shown to be a part of human diet e.g. in bovine milk, eggs, and even lamb meat [King et al., 1998; Bannwart et al, 1988, Kuhnle et al., 2008], it is important to widen the research in to this field, as well.

1.1 ISOFLAVONOIDS

Isoflavonoids (Figure 1) are a class of natural products, present mainly in Leguminosae, the third largest family of flowering plants. By the end of 2004 more than 1600 compounds were known [Veitch, 2007].

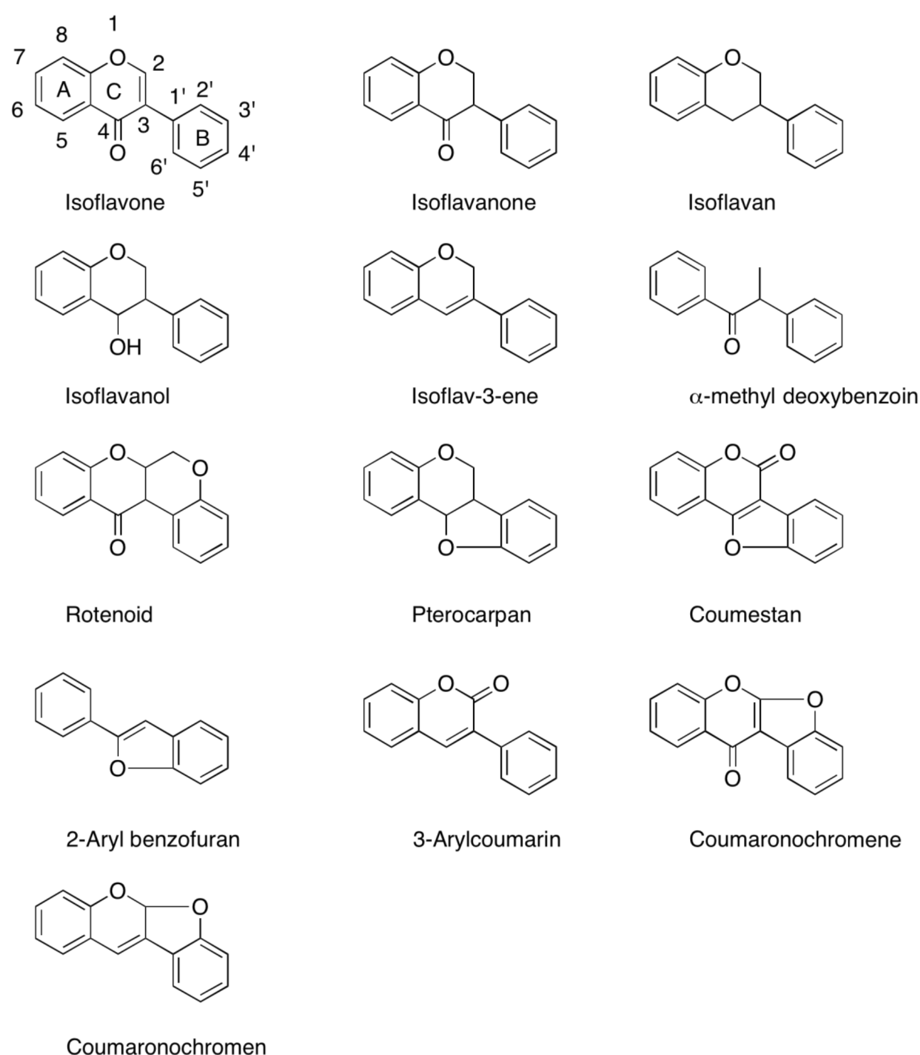
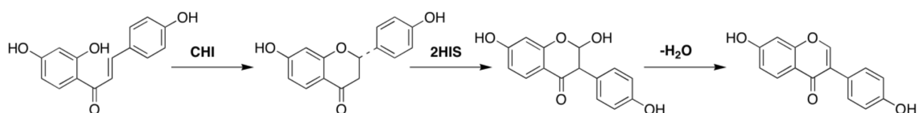


Figure 1 Isofavonoid structures.

Isoflavonoids share a common 3-phenylchromone structure (C6-C3-C6), which is derived from the 2-chromone structure by rearrangement (Scheme

1). They can be divided into several subgroups based on their structure and biogenesis. The most abundant isoflavonoid subclass consists of isoflavones, which have 3-phenyl-4H-1-benzopyran-4-one as the parent ring system. Isoflavonoids are found almost exclusively in leguminous plants such as soy and clover.



Scheme 1 Biosynthesis of daidzein. CHI, chalcone isomerase; 2HIS, 2-hydroxyisoflavanone synthase [Veitch, 2007].

1.2 BIOLOGICAL SIGNIFICANCE OF ISOFLAVONOIDS IN PLANTS

In plants isoflavonoids function as phytoalexins. They protect plants from various environmental stress factors such as disease and pesticides. Some isoflavonoids protect plant cells from radiation due to their strong absorption in the UV range.
Phytoalexins are traditionally defined as compounds plants synthesise de novo when they are attacked by a pathogen. Simple isoflavones (Figure 2) such as daidzein (DA), genistein (GEN), formononetin (FO), biochanin A (BA) and coumestrol in the sub-class of coumestans (COU) are common compounds in legumes. They are often considered as phytoalexin precursors, and they are known to prevent the growth of some microbes. On the other hand, it has been shown that DA, GEN, and COU increase the growth of some beneficial microbes.

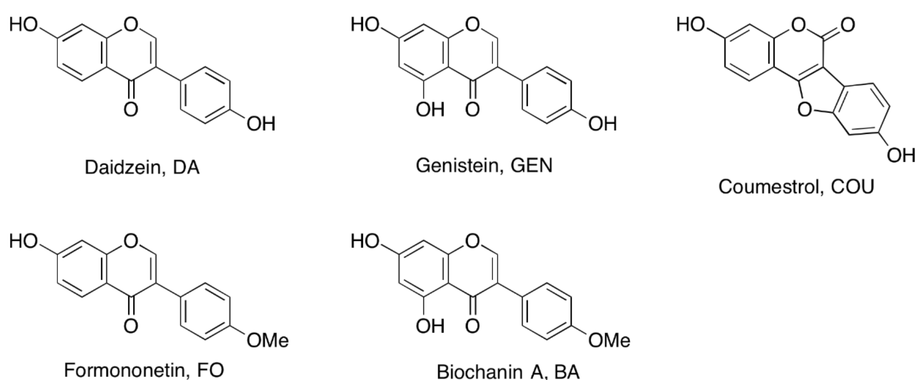


Figure 2 Common isoflavones and coumestrol.

1.3 ANALYSIS OF ISOFLAVONOID PHYTOESTROGENS

1.3.1

The predominant method of analysis of phytoestrogens in general is HPLC. The main advantage is the ease of use. Samples do not necessarily require time-consuming pre-treatment and may, in principle, be injected into a system right after the extraction and analysed as such. The problem has been to achieve a good enough resolution and sensitivity since phytoestrogen levels are usually quite low, especially in biological fluids [Hoikkala et al., 2003].

UV or UV-DAD detectors used to be most commonly used for detecting analytes, but new methods have been introduced. For example, coulometric electrode array detection has been utilized for the quantitation of the phytoestrogens in human fluids. Coulometric array detection uses a porous flow-through carbon graphite working electrode. It has a high surface area, which allows efficient electrolysis. This results in a highly reproducible total peak area (coulombs) and very low susceptibility to the loss of signal from adsorptive effects. This technique has significant advantages in the detection of phenolic phytochemicals due to the inherent sensitivity, selectivity and linear response range of electrochemical detectors. Also, because of the unique properties of the coulometric electrode, resolution of co-eluting solutes can be achieved based on small differences in their oxidation-reduction behaviour [Penalvo and Nurmi, 2006].

The breakthrough in the application of HPLC-MS has come from the development of two interfaces, the electrospray ionization (ESI) interface and the heated nebulizer atmospheric pressure chemical ionisation (HN-APCI) interface [Prasain et al., 2004]. HPLC-MS has the advantage over GC-MS in that it is not necessary to prepare volatile derivates and that conjugated forms can be analysed as easily as the aglycones. ESI-MS is well suited for the analysis of phytoestrogen conjugates such as sulfates and glucuronides in human fluids. Thermally labile conjugates form molecular ions in ESI. With APCI interface, however, these physiological conjugates decompose since they are not stable enough. Instead of forming molecular ions, they form aglycone ions [Barnes et al., 1998].

1.3.1.1 UHPLC

New generation HPLC instruments allow much higher pressure than previously and therefore also the use of smaller particle size in columns. Traditional instruments can operate at pressures up to 400 atm with particle sizes around 3 μm. With new instruments, the pressure can be as high as 1000 atm and particle size < 2 μm. The method is called UHPLC or UPLC (Ultra High Performance Liquid Chromatography). The latter, however, is a trademark used by the Waters corporation. Theoretically higher pressure and

smaller particle size means better resolution and faster and more sensitive analysis.

Churchwell et al. have compared traditional HPLC-ES/MS/MS with UHPLC-ES/MS/MS using tamoxifen and soy isoflavones and metabolites as model compounds [Churchwell et al., 2005]. They noticed sharper peaks and hence better S/N ratios with UHPLC. The analysis time also decreased from 10 mins to 3.5 mins when compared to their previous method. Similarly, they were able to analyse phytoestrogen glycosides from soy supplement with precursor ion scanning in 5 minutes compared to 23 minutes with conventional HPLC.

Farré et al. compared HPLC, UHPLC in the analysis of phytoestrogens in water samples [Farre et al., 2007]. DA, GEN and BA were analysed from spiked and non-spiked water samples with MS/MS detection. They were able to shorten the analysis from 45 minutes to 15 minutes with UHPLC. Also detection limits were lowered.

Oleszek et al. used UHPLC for the analysis of isoflavones in the aerial parts of different Trifolium species [Oleszek et al., 2007]. They used daidzin as a reference compound for the quantitation of isoflavones with UV-DAD detector in a 10 min run.

Kledjus et al. have identified isoflavones and their glycosides from plant samples with UHPLC-UV-MS [Klejdus et al, 2005]. Samples were extracted with a supercritical fluid before analysis. Later on they reported a rapid-resolution technique for the analysis of isoflavones in plant extracts [Klejdus et al, 2007]. The analysis time was less than one minute per sample with UV-DAD detection.

Muth et al. used UHPLC to differentiate between glycocojugates of Gen and other isoflavones and flavones [Muth et al., 2008].

Vila-Donat et al. used UHPLC coupled to MS/MS-detection for the determination of isoflavones and their glycosides in legumes [Vila-Donat et al., 2015]. The method was used to analyse 48 lentil samples. Samples were ground and extracted (MeOH-H2O, 80:20 v/v) and subjected to freezing clean-up to precipitate proteic and lipidic residues. The analysis time was 18 minutes per sample.

Prokudina et al. developed a UHPLC-ESI-MS method for the analysis of 15 isoflavonoids among 26 phenolic compounds in plant material [Prokudina et al., 2012]. The analytes were quantified using deuterated internal standards ($[^{2}H_{6}]$naringenin, $[^{2}H_{4}]$genistein) and $[^{13}C_{3}]$daidzin. The application of the method was demonstrated by the analysis of mung bean (Vigna radiata) sprout extract. The lyophilised sample was homogenised in 50 % EtOH solution containing internal standards using ball mill. Samples were further sonicated, centrifuged and filtered before injection.

1.3.1.2 HPLC with UV/UV-DAD detection

Franke and co-workers have developed a method for the analysis of isoflavonoid phytoestrogens in human urine [Franke et al., 1995]. In successive papers they have extended the method for determinations in foods and in human milk [Franke and Custer, 1996; Franke et al, 1998a; Franke et al. 1998b], with a few changes to the original method. DA, GEN, FO, BA, COU and their metabolites such as equol (EQ) and O-desmethylangolensin (*O*-DMA) were analysed after isolation by solid-phase-extraction (C-18 SPE). Analytes were isolated by solid-phase-extraction (C-18 SPE) and then analysed by HPLC. Samples were hydrolysed enzymatically by incubating for 24 h at 37 ^{o}C, after mixing thoroughly with a freshly prepared mixture of acetate buffer, ascorbic acid and glucuronidase/sulfatase. The samples were then centrifuged and the supernatants used for injection to HPLC. A NovaPak C-18 reversed-phase column, coupled to an Adsorbosphere C18 direct-connect guard column was used. Gradient elution with a flow rate at 0.8 ml/min used two mobile phases (acetonitrile, then acetic acid-water (10:90)). Analytes were identified by their retention times, after a UV scan (a fluorometric detection for COU excitation 340 nm, emission 418 nm). The detector used was a dual wavelength diode-array (260 nm; 280 nm for EQ and 342 nm for COU elution). The detection limits were 5-780 nM (for 20 ml injection). For estimation of recovery, flavone was used as an internal standard.

Supko et al. [Supko and Phillips, 1995] reported a procedure involving isocratic reversed-phase HPLC with UV detection for the determination of GEN in biological matrices with very high recoveries (plasma mean: 94.8%, urine mean: 91.4%). The analyte was extracted with tert-butylmethyl ether (MTBE). The extract was centrifuged and supernatant recovered. After evaporation of the solvent, the residue was dissolved in a solution of methanol and ammonium acetate buffer (0.05 M, pH 4.5). An isocratic elution with Nova-Pak C-8 reversed phase column was employed. The effluent was monitored at 260 nm. The lowest concentration of GEN was 0.02-1.00 mg/ml with a sample volume of 50ml. Simply increasing the sample size, without otherwise modifying the assay procedure, increased the sensitivity of detection. A tenfold improvement was afforded by increasing the sample size to 250 ml.

Kulling et al. have reported several methods for the oxidative in vitro metabolism studies of DA and GEN [Kulling et al., 2000]. The metabolites were identified using HPLC with diode array detection or atmospheric pressure ionization electrospray mass spectrometry and also GC/MS. Samples for the HPLC analysis were prepared by extracting the liver microsome mixture with ice-cold ethyl acetate. After evaporation of the organic solvent the samples were re-dissolved in methanol and subjected to HPLC analysis.

In our studies we have used HPLC with UV-DAD detection together with fluorescence detector to study the levels of isoflavonoid phytoestrogens (I, II). Bovine milk samples were analysed after diethyl ether extraction and centrifugation followed by solid phase extraction.

1.3.1.3 HPLC with MS detection

Coward and coworkers have developed a method using reversed-phase HPLC-MS [Coward et al., 1996; Sfakianos et al., 1997]. The method allows the construction of a mass/intensity map of several isoflavonoid metabolites in a single 20-min analysis. The sample was first extracted with a Sep-Pak C-18 cartridge and an internal standard was added. The sample was then analysed with HPLC-MS or hydrolysed enzymatically and extracted again with Sep-Pak cartridges. Analyses of isoflavonoids and other phytoestrogens were carried out on an Aquapore C8 reversed-phase HPLC column using a linear gradient of 0%-50% acetonitrile in 10 nM ammonium acetate (pH 6.5), over 10 min at flow rate of 1 ml/min. The solute was introduced into MS via the HN-APCI interface operating in either the positive or negative mode.

Doerge with co-workers have developed a simple and sensitive LC/ESI-MS method using deuterated internal standards (daidzein-d3 and genistein-d4) for the determination of daidzein and genistein and their conjugates in rat blood [Holder et al., 1999]. Serum and plasma samples in acetonitrile were vortexed, sonicated and centrifuged to remove precipitated proteins. After selective hydrolysis, the aglycones were extracted into ethyl acetate. The samples were then evaporated and reconstituted in methanol and diluted with water before analysis by HPLC. Chromatography was performed using a C-18 column with isocratic elution. The detection limits for daidzein and genistein were approximately 5nM (50 ml of rat blood). The recoveries of the analytes were approximately 85%. Later the method was modified further to eliminate laborious cleanup procedures [Chang et al., 2000; Doerge et al., 2000]. A restricted-access chromatographic medium was used for the automation of the analysis. Enzymatically hydrolysed samples were subjected to centrifugal filtering before loading onto the trap cartridge for sample cleanup. This procedure gave >80% recoveries. Also rat tissue was analysed for the determination of the genistein tissue distribution. Thawed tissue was homogenized and extracted with methanol by sonicating the suspension and hydrolysed enzymatically when total genistein levels were determined. Lipids were extracted from fatty tissues into hexane. After centrifugation, the supernatants were loaded onto Oasis HLB solid phase extraction cartridges before being subjected to LC-ES/MS analysis. Limits of detection varied depending on the tissue matrix and were in the range of 0.04-0.09 pmol/mg (LC-ES/MS) and 0.01-0.03 pmol/mg (MS/MS). Recoveries of genistein from tissues ranged from 40 to 78%.

Cimino et al. developed an LC method for the separation of isoflavones and their metabolites from urine, establishing the concentrations with HPLC-APCI-MS [Cimino et al., 1999]. For the extraction from urine, ammonium acetate was added to each sample and the total isoflavones were extracted in diethyl ether, and the organic layers were evaporated to dryness with nitrogen. The free isoflavones were extracted directly from the sample. The sulfates and glucuronides were subjected to enzymatic hydrolysis by β-glucuronidase or sulfatase or both when total content was analysed. After evaporation the solid

sample was dissolved in the mobile phase, which consisted of two different solvent compositions. The column was an RPamide-C16. Isoflavones were eluted with a linear gradient. Detection sensitivity of carbonyl containing isoflavones was increased by the infusion of 15% ammonium hydroxide at 0.14 ml per min to the eluate. Analytes were detected by mass spectrometry using negative single ion monitoring. Detection limits for all analytes, except for equol, were 5 ng/ml of urine. The detection limit for equol is 200 ng/ml of urine.

Clarke et al. have developed an isotope dilution liquid chromatography-tandem mass spectrometry method (LC-ESI-MS/MS) for the identification of phytoestrogen conjugates from urine [Clarke et al., 2002]. It allows the detection of intact phytoestrogen conjugates as well as of free aglycones. The method uses isotopically labelled [$^{13}C_3$] daidzein and [$^{13}C_3$] genistein as internal standards. Furthermore, it allows the analysis of IFs without any sample pre-treatment, i.e. urine samples were injected directly onto the column. The limits of detection were <50 ng/mL. Due to the lack of standards for the majority of conjugates the amounts were determined semiquantitatively. This method makes the assumption that all positional isomers of given conjugate class (glucuronides, sulfates, etc) will have similar molar response factors.

Grace et al. have described a similar method for the quantification of IFs in serum [Grace et al., 2003a]. They use triply ^{13}C-labelled internal standards of GE, DA, EQ, and *O*-DMA . The analysis is done using LC/MS/MS. The serum samples were hydrolysed using *Helix pomatia* after which the aglycones were extracted from the hydrolysate with solid phase extraction using C-18-E SPE cartridges. The samples were applicated to a conditioned cartridge and washed with 5% methanol. The aglycones were eluted with 1:1 ethyl acetate-acetonitrile. The eluates were dried and redissolved in 40% methanol before injecting them onto HPLC. The method was further developed for high throughput quantification of phytoestrogens human urine and serum. This was achieved via the use of 96-well plate sample extraction and LC/MS/MS analysis incorporating column switching [Grace et al., 2007].

Piskula et al. have studied the absorption of DA and GEN aglycones and glucosides in rat [Piskula et al., 1999]. The determination of isoflavones in rat plasma was conducted by HPLC. After enzymatic hydrolysis, samples were extracted with methanol/acetic acid. After centrifugation, the supernatant was diluted with water and analysed using a C-18 column and an amperometric electrochemical detector. For the blood plasma analysis, the isolation procedure is somewhat more complicated. After hydrolysis, plasma was extracted three times with methanol and centrifuged. Supernatant was diluted with water and extracted with chloroform. After extraction, the methanol/water phase was evaporated and residue was redissolved in methanol. The sample was then centrifuged at 4 °C and supernatant was evaporated again. Next, the residue was dissolved in water and after sonication extracted with Sep-Pak C-18 cartridge. After further evaporation, the sample

was dissolved in methanol and subjected to HPLC analysis. Again, a C-18 column was used with diode array detector.

1.3.2 GC ANALYSIS

The analysis of phytoestrogens from biological samples often requires pre-purification. Samples are first extracted from the sample matrix by organic solvents such as diethyl ether or ethyl acetate. Also C-18 cartridges have been used for solid phase extraction. For the enhancement of sensitivity, the removal of interfering constituents such as steroids is required. This can be done by ion exchange chromatography on DEAE and QAE Sephadex. Also various conjugates and aglycones can be separated with the same procedure.

When GC is employed for the analysis, samples also require hydrolysis and derivatisation. Since sample pre-treatment involves several steps it is necessary to use internal standards to correct for losses during sample pre-treatment. Internal standards should be chemically analogous to the samples to be analysed so that they will behave similarly during the extractions and isolations. This is usually achieved by using different homologues or compounds carrying stable isotopic labels such as deuterium or carbon-13. It should be noted that when the analytes are derivatised as their silyl ethers for the GC-MS analysis the combined effect of the heavier stable isotopes of silicon and carbon is such that considerable M+1, M+2 etc. peaks appear in the mass spectra. Thus the contribution of a single synthetically introduced isotopic label, either D or ^{13}C, will be almost lost. This is why our group has developed methods for the synthesis of stable polydeuterated standards that contain a minimum of three D atoms and in some cases up to eight D atoms, which do not back exchange [Wahala and Rasku, 1997; Rasku et al, 1999; Salakka and Wahala, 2000]. The mass spectral peak to be used as reference in quantitation will then be free from interference from the natural abundance C-13 and heavier silicon atoms present in the TMS ether derivatives.

To avoid the creeping of analytes, all glassware is usually silanilazed before use by a 10% solution of dimethylchlorosilane in heptane [Liggins et al., 2000] (Liggins et al. 2000) or a 1% solution in toluene [Adlercreutz. et al., 1993].

Phytoestrogens are present in biological fluids usually as the glucuronide and/or sulfate derivatives. Adlercreutz et al. have used a β-glucuronidase solution from Escherichia coli K12 in acetate buffer to hydrolyse glucuronides [Adlercreutz et al., 1995a]. Sulfates were solvolysed in dimethylformamide-6M HCl-dichloromethane solution overnight at 37 °C. Samples may also be hydrolysed with β-glucuronidase/sulfatase enzyme juice extracted from the digestive juice and hepatopancreas of the snail *Helix pomatia*. Because the juice may also contain small amounts of lignans and isoflavonoids, it has to be purified before use. Mazur et al. [Mazur et al., 1996] have used 1% charcoal in 0.66 M acetate buffer shaked overnight at room temperature. Solid-phase extraction C-18 cartridges [Setchell et al., 2001] and Amberlyte XAD-2 column [Morton et al., 1994] have also been used for purification. Samples are first

hydrolysed if the total phytoestrogen content is to be analysed. Another way is to separate aglycones and conjugates from each other or even different conjugates from each other before the hydrolysis.

Trimethylsilyl (TMS) derivatives are prepared either with pyridine-hexamethyldisilazane-trimethylchlorosilane (Py-HMDS-TMSCl) (9:3:1) (QSM) or BSTFA (N,O-bis-(trimethylsilyl)-trifluoroacetamide). For the t-butyldimethylsilyl (TBDMS) ethers, N-methyl-N-TBDMS-trifluoroacetamide and TBDMS chloride in acetonitrile are used [Setchell et al., 2001]. BSTFA being a strong silylating agent may cause the formation of artefacts as reported. In addition to silylation of free hydroxyl groups also carbonyl groups may be silylated in the enol form [Wahala and Rasku, 1997; Heinonen et al., 1999]. Furthermore derivatisation of the isoflavone metabolites dihydrodaidzein or dihydrogenistein in dry pyridine may yield a C-ring cleavage product referred to as dehydro-*O*-DMA and 6´-OH-dehydro-*O*-DMA, respectively. Derivatisation of dihydrogenistein with QSM produced 6´-OH-dehydro-*O*-DMA only as a minor product [Heinonen et al., 1999].

1.3.2.1 GC/MS

Kelly and co-workers have described a simple method for determining total isoflavones and their metabolites together with ENL in human urine [Kelly et al., 1993; Joannou et al., 1995]. Urine samples were first hydrolysed with β-glucuronidase aryl sulfatase. The samples were then extracted with diethyl ether, the organic phase evaporated and the residue dissolved in ethanol. The analytes were isolated by partition chromatography on Sephadex LH-20 and derivatised prior to analysis by GC and GC-MS. ^{3}H-estradiol glucuronide and estradiol were used as internal standards. Derivatisation by BSTFA in this method proved later to have produced metabolite artefacts [Heinonen et al., 1999].

Setchell et al. have reported a method for determining the isoflavones DA, GEN, GL, BA and FO from human plasma and urine using two stable isotopically labelled internal standards and an isoflavone homologue [Setchell et al., 1997, Setchell et al., 2001]. Total and free isoflavones were identified separately. First ^{13}C daidzein, ^{13}C genistein and 4´,7-dihydroxyflavone were added as internal standards to plasma samples. For total isoflavones, samples were extracted with C-18 BondElut cartridges. Samples were then hydrolysed with β-glucuronidase/sulfatase from *Helix pomatia*. Hydrolysed samples were extracted again with C18 BondElut cartridges. Isoflavones were separated from neutral compounds and purified with TEAP-Sephadex LH-20 in the hydroxide form and derivatised. Free isoflavones were determined as above but without the hydrolysis step. TBDMS derivatised samples were analysed by GC-MS.

Setchell and co-workers have analysed human serum and urine to study the pharmacokinetics of DA and GEN using ^{13}C-labeled tracers [Setchell et al., 2003]. The analysis was performed by GC-MS for the serum and HPLC-MS

for the urine. Dihydroflavone was added to a serum sample as an internal standard and the sample was then diluted with buffer. Isoflavones and their conjugates were recovered using C-18 solid phase extraction. The samples were then hydrolysed using *Helix pomatia* digestive juice. After hydrolysis the aglycones were isolated by a similar C-18 solid phase extraction. The samples were then further purified by TEAP Sephadex LH-20 prepared in the [OH-] form and packed in methanol. The phenolic compounds were recovered by elution of the gel bed with methanol saturated with carbon dioxide. The aglycones were derivatized by the addition of acetonitrile and N-methyl-N-t-butyldimethylsilyltrifluoroacetamide in 1% t-butyldimethylchlorosilane.

Morton et al. have described a GC-MS method for determining the total phytoestrogens DA, GEN and EQ in human plasma [Morton et al., 1994, 1997]. A plasma sample was hydrolysed with β-glucuronidase and deuterated internal standards were added. The hydrolysate was extracted with diethyl ether and the organic phase was collected. Phytoestrogens were isolated with TEAP-Sephadex LH-20 in the hydroxide form as described by Setchell et al. [Setchell et al., 1976]. BSTFA-derivatised samples were then analysed with GC-MS using single ion recording (SIR). Later the method was modified for the analysis of prostatic fluid and blood [Morton et al., 1997]. Phytoestrogens were isolated with Sephadex LH-20 as described by Kelly et al. [Kelly et al., 1993]. For serum analysis ethyl acetate was used for extraction [Morton et al., 1999].

Grace et al. have reported a simple method for analysing isoflavones and lignans from urine by GC/MS using $^{13}C_3$-labeled internal standards [Grace et al., 2003b]. To a thawed urine sample, acetate buffer was added together with a mixture of $^{13}C_3$-labeled standards in methanol. The mixture was then hydrolysed using H. pomatia. Aglycones were separated by solid phase extraction. Sample was applicated to a conditioned column and washed with 5% methanol. The aglycones were eluted with 1:1 ethyl acetate-acetonitrile. The dried eluates were derivatized by adding anhydrous pyridine and BSTFA with 1% TMS. Anthraflavic acid was added to act as an internal standard for *O*-DMA and GLY.

We have used GC/MS for the qualitative study of isoflavone metabolism (IV). The urine samples were hydrolysed with *Helix pomatia* then extracted with diethyl ether and purified with Sephadex LH-20. Samples were analysed as trimethylsilyl derivatives.

1.3.2.2 ID/GC/MS/SIM

Adlercreutz et al. have described a method for the quantitative determination of phytoestrogen in biological samples. They use isotope dilution gas chromatography - mass spectrometry with selected ion monitoring (ID/GC/MS/SIM). Stable deuterated derivatives of the compounds to be analysed are added to the sample at the start of the procedure. Recoveries are calculated by monitoring the mass peaks of analytes

and co-eluting deuterated samples. The lignans ENL, END and the isoflavonoids DA, EQ and *O*-DMA were first analysed from human and chimpanzee urine using different modifications of the method [Adlercreutz et al., 1986].

A slightly different method, developed originally for determining the whole estrogen profile in urine has been applied for the analysis of phytoestrogens in urine [Adlercreutz et al., 1991a, 1991b] and plasma [Adlercreutz et al., 1993]}. Plasma samples were first extracted with Sep-Pak C-18 cartridges before ion exchange chromatography and solvolysed after evaporation of the solvent with HCl (such as HCl). Free and conjugated phytoestrogens were separated with DEAE-Sephadex in the acetate form. The phytoestrogen fraction originally contained free and mono- and disulfated (now unconjugated after solvolysis) phytoestrogens. The following fractions contained mono and diglucuronides and solvolysed sulfoglucuronides. This fraction was purified further with Sep-Pak C-18 cartridges. Deuterated internal standards were then added to both samples and the conjugated phytoestrogen fraction was hydrolysed with β-glucuronidase. Due to unavailability of conjugated internal standards the recoveries until this step were corrected using radioactive standards added in the beginning of the entire procedure. The standards used for this purpose were ^{3}H-estrone sulfate and ^{3}H-estrone glucuronide. Free and conjugated fractions were then subjected to QAE-Sephadex chromatography in the acetate form. The first fraction contained all estrogens and ENL, END, MAT and EQ. The second fraction contained *O*-DMA, DA and GEN. Estrogens from the first fraction were further separated with QAE-Sephadex in the carbonate form. After this samples were derivatised before GC/MS/SIM analysis. The method was modified for the analysis of phytoestrogens in plasma [Adlercreutz et al., 1993]. Estrogenic compounds (15 endogenous estrogens and phytoestrogens) may be analysed by one run by this method. It was utilized by Lampe and co-workers to compare the effect of vegetable, fruit and legume consumption on urinary phytoestrogen isoflavonoid and lignan excretion [Hutchins et al., 1995; Kirkman et al., 1995; Lampe et al, 1998, 1999, 2001].

The method has been developed further to determine the pattern of conjugation of the phytoestrogens in human urine [Adlercreutz et al., 1995a]. The first DEAE-AcO- chromatography is modified to separate the free fraction, mono- and disulfate as well as mono-, di -and sulfoglucuronide fractions. The method has been applied for the determination of unconjugated lignans and isoflavonoids in human faeces [Adlercreutz et al., 1995b]. Metzler et al. have modified the method for determining metabolites of mammalian lignans in human urine and further to determine metabolites of ENL and END in rat bile and urine [Jacobs et al., 1999; Niemeyer et al., 2000]. The method has also been applied by Kurzer et al. to determine phytoestrogens in human urine [Xu et al., 1998, 2000].

1.3.3 IMMUNOASSAYS

The methods described above are not suitable for screening purposes in large populations. In addition, they are not sensitive enough for the assay of unconjugated phytoestrogens in plasma. These disadvantages have led to the application of a new analytical method based on immunoassay.

In immunoassay an antigen and an antibody interact reversibly to form a soluble antigen-antibody complex. Labelled and unlabelled antigens compete for binding to the limited number of antibody binding sites. The greater the quantity of unlabelled antigen, the less labelled antigen is bound. The concentration of unlabelled antigen is derived from the extent to which it competitively inhibits the binding of the radioactive antigen to a specific antibody, the method being standardised with known concentrations of unlabelled antigen.

A derivative of the molecule (hapten) for which the assay is prepared is coupled to a carrier molecule (usually bovine serum albumin, BSA) and the conjugate is used for immunisation. Only three isotopes appear as suitable candidates for labelled antigen, called tracer, in radioimmunoassay: ^{131}I, ^{125}I and ^{3}H, although ^{131}I is less useful on account of its short half-life [Dwenger, 1984]. For fluoroimmunoassay (FIA), a europium labelled or other appropriate tracer is synthesised. Radioactivity or fluorescence of the fractions is measured and quantitative results are obtained by comparing the counts against a standard curve. Qualitative and quantitative evaluation of an antibody used for radioimmunoassay is based on two criteria. Specificity is determined by cross-reaction with antigen analogues, and affinity is determined by the measurement of affinity constants. Immunoassays usually provide an increase in sensitivity compared to GC-MS or HPLC but sometimes a decrease in selectivity since cross-reactivity is possible.

1.3.3.1 RIA

A radioimmunoassay for the determination of formononetin was established by Wang, employing rabbit antisera against formononetin-7-BSA and the tritium-labelled homologous radioligand conjugate formononetin-7-([^{3}H]-leucine) [Wang, 1998]. This RIA procedure enabled the quantification of 200 pg/mg of plasma or 50 pg/mg of mammary tissue. Lapcík et al. have developed a radioimmunoassay procedure to determine DA in plasma, serum and urine using daidzein-4-BSA for immunization and an ^{125}I labeled tracer [Lapcik et al., 1997]. DA was measured either directly or after extraction in ether. The working range of the assay was 1.5-200pg/tube. The method did not discriminate between DA and its 4'-methoxy derivative FO which caused almost 60% cross-reaction. This is because the antisera prepared via the 4'-position recognize also 4'-methoxy derivatives. In the same way antisera prepared via the 7-position recognize 7-methoxy derivatives [Lapcik et al., 1999]. Also cross-reaction with 4'-sulfates and –glucuronides probably occurred since daidzein values after extraction were only 8% of those obtained

with direct serum analysis. Adlercreutz together with Lapcík et al. established two radioimmunoassays (RIA) for genistein based on polyclonal antibodies against genistein-4-O-(carboxymethyl) ether-bovine serum albumin and against genistein-7-O-(carboxymethyl) ether-bovine serum albumin conjugates [Lapcik, 1998, 1999; Uehara et al., 2000]. The sensitivity of the assays was 1.2 and 2.8 pg/tube respectively.

Time–resolved fluoroimmunoassay (TR-FIA) was developed for the rapid analysis of ENL in human plasma, using a europium chelate as a label, by Adlercreutz et al. [Adlercreutz et al., 1998]. It uses 5'-O-carboxymethoxyenterolactone haptens for the immunization and production of tracer [Uehara et al., 2000]. It seems to be highly specific since no cross reactions were observed for phytoestrogens except to a small extent with enterodiol (0.28%). The minimum amount of enterolactone distinguishable was 2.1 pg/20μl. The analysis method is very fast. A batch of one hundred samples can be completed in four hours [Lapcik, 1998]. It has a 10- to 100-fold increase in sensitivity and assay range compared to conventional enzyme immunoassay (EIA) and fluoroimmunoassay (FIA) methods. It was, however, noticed that the method gave too high values for plasma enterolactone. The method was modified by lowering the amount of hydrolysis reagent thus reducing the absolute amount of sulfatase, which caused the error in the values [Stumpf et al., 2000]. Later, a method for determining DA and GEN in plasma and urine was developed [Wang et al., 2000; Uehare et al. 2000]. Phytoestrogens were measured from hydrolysed samples. The minimum amount for daidzein and genistein, distinguishable from zero sample were 1.8 pg/20 ml and 3.2 pg/20 ml, respectively. Kohen et al. reported a highly specific TR-FIA method for the measurement of DA in urine [Kohen et al., 1998]. DA was analysed in urine after hydrolysis with β-glucuronidase.

1.3.3.2 ELISA

Enzyme-linked immunosorbent assay (ELISA) was also developed for the analysis of FO, DA, EQ, BA and GEN [Bennetau-Pelissero et al., 2000]. Seven carboxylic acid haptens were produced and used in development of the assay suitable for assays in vegetable matter and biological fluids. IC_{50} values of the standard curves were between 0.8 nl/mL and 20 ng/mL.

1.3.4 OTHER METHODS

A simple and rapid method for the analysis of isoflavones in food samples has been developed by Wang and Sprons [Wang and Sporns, 2000]. They have used the MALDI-TOF technique to detect DA, GEN and GLY together with their various conjugates in soy products. By selecting the proper matrix, this technique provides an isoflavone profile of food sample in few minutes and

can be used for both quantitative and qualitative analysis especially in conjunction with other techniques such as HPLC.

Lojza et al. analysed isoflavones in soybeans using direct analysis in real-time (DART) ion source coupled to a high resolution orbitrap mass spectrometer [Lojza et al., 2012]. DART is an APCI-related technique which employs a glow discharge for the ionization. It is a soft ionization technique suitable for a wide range of compounds. In their study Lojza et al. developed a rapid method for the specific determination of isoflavones and their glycosides in soybeans. Homogenized sample were extracted using 80% MeOH and optionally hydrolysed to aglycone form with 6 M HCl under reflux. As a comparative method they used UHPLC-ESI-orbitrap for the analysis of the samples.

Capillary electrophoresis is a powerful technique, affording rapid and high-resolution separations (104-106 theoretical plates) while requiring just femtomoles of sample. The utility is greatly enhanced by mass spectrometric detection, particularly with electrospray ionisation, which can be used to produce ions from thermally labile, non-volatile compounds such as conjugated phytoestrogens. It has been used to determine isoflavones from soybean seeds using UV detection [Aussenac et al., 1998]. Also a CE-MS study of isoflavones has been published [Aramendia et al., 1995]. One potential limitation with CE concerns the low sample loads owing to the small inside diameter of the capillaries, the sample injection volumes being confined to the low nanolitre and low picomole range in order to maintain high separation efficiency. Hence the concentration detection limit for CE/MS is often unsatisfactory.

1.3.5 CONCLUDING REMARKS OF ANALYSIS

Quantitative analyses of isoflavonoids in plants, foods and biological fluids are important in epidemiological, pharmacological, phytoestrogenic, chemotaxonomic, breeding, and other biochemical studies. Isoflavonoids are present in plants as mixtures of aglycones and glycosides, and in biological fluids as mixtures of aglycones with glucuronides, sulfates or with sulfates and glucuronides together. In addition, initial extracts may contain many structurally related compounds. These include flavonoids, steroids and other polyphenolic compounds. Methods for quantitation must therefore be capable not only of separating isoflavonoids from other compounds but also of distinguishing between the various isoflavonoid structures. Phytoestrogens are presently identified and determined mainly by GC-MS and HPLC, techniques that are time-consuming and labour-intensive and require sophisticated and expensive instrumentation. More effective and economical methods for the monitoring of human exposure to phytoestrogens should be specific and sensitive and allow large numbers of samples to be processed reliably and rapidly. Immunoassay offers the advantages of speed, sensitivity and high throughput, with the potential for automation.

Gas chromatography (GC) combines the advantage of high separation capability with the disadvantages of the need for prepurification, fractionation, hydrolysis and derivatisation of samples. Gas chromatography - mass spectrometry (GC-MS) is the basis for the majority of existing methods for the quantitative analysis of isoflavones and their metabolites in biological fluids, including urine, plasma, and faeces. GC-MS in the selected ion-monitoring mode employs deuterated internal standards to compensate for losses during the isolation process.

Moreover, HPLC methods have been developed to allow the determination of a variety of isoflavonoids, including aglycones and conjugated isoflavonoids. Use of fluorescence detection and photodiode array detection in place of UV detection is a useful way of increasing the sensitivity. Whilst UV photometry at 260 nm can detect nanogram quantities of DA and GEN, the limit of electrochemical detection is about ten picograms. Advantages of HPLC-MS over GC-MS analysis include higher precision, less manipulation, and applicability to non-volatile components with direct injection of the liquid sample.

1.4 METABOLISM IN HUMANS AND IN RODENTS

Metabolism of isoflavones plays an important role since the activity of the compound is altered after structural modifications. It may well be that many of the so-called estrogenic effects of phytoestrogens are in fact due to their metabolites and not the intact plant derived isoflavone.

Isoflavones usually occur in conjugated form in plant material. The main source of isoflavones for human is leguminous-based food products. The predominant isoflavones in soy are DA, GEN and GLY. Another source, which has been studied, is red clover supplements that have been marketed to provide beneficial health effects of isoflavones without change in the original diet. The isoflavone profile of red clover differs from that of soy and has to be taken into account when the metabolism is considered. The main isoflavones in red clover are FO and BA. Isoflavones are present in plants mainly as 7-β-D-glycosides of glucose and 6’’-O-malonylglucose. Once ingested they undergo several transformations along their passage through the gastrointestinal tract.

Before isoflavones can be absorbed they must be deconjugated. After deconjugation isoflavones are metabolized by liver enzymes or intestinal bacteria to the so-called phase I metabolites. Before excretion they are conjugated to β-gluronides and sulfate esters to form phase II metabolites.

D’Alessandro et al. and Aura have reviewed the metabolism of isoflavone phytoestrogens and plant phenolics in general [D'Alessandro et al., 2005; Aura, 2008].

1.4.1 PHASE I OXIDATIVE METABOLISM

After absorption, isoflavone aglycones are metabolised in liver microsomes by cytochrome P450 enzymes [Roberts-Kirchhoff et al., 1999]. Hu et al. indentified different isoforms of cytochrome P450 responsible for the metabolism of genistein [Hu et al. 2003]. 3'-Hydroxygenistein was found to be the main metabolite and CYP1A2 the main isoform. Furthermore, they confirmed the demethylation of FO, BA, prunetin (PR) and 7,4'-isoflavone to DA and GEN. In an in vivo study several new oxidised metabolites were found in human urine after soy based diet [III]. The isoflavones daidzein, genistein and glycitein were converted to hydroxylated isoflavones. The identification of urinary metabolites after dietary red clover supplements revealed new metabolites in human subjects [Heinonen et al., 2004].

Kulling et al. has studied the oxidative metabolism of daidzein and genistein in vitro using rat liver microsomes [Kulling et al., 2000] and later in vivo from human urine and in vitro using human liver microsomes [Kulling et al., 2001]. Several new hydroxylated metabolites were found when DA and GEN were incubated with liver microsomes. New metabolites were identified partly using authentic standards and partly by interpreting the mass fragmentation pattern of the analytes. Similar results were obtained when human urine was analysed after a soy-based diet.

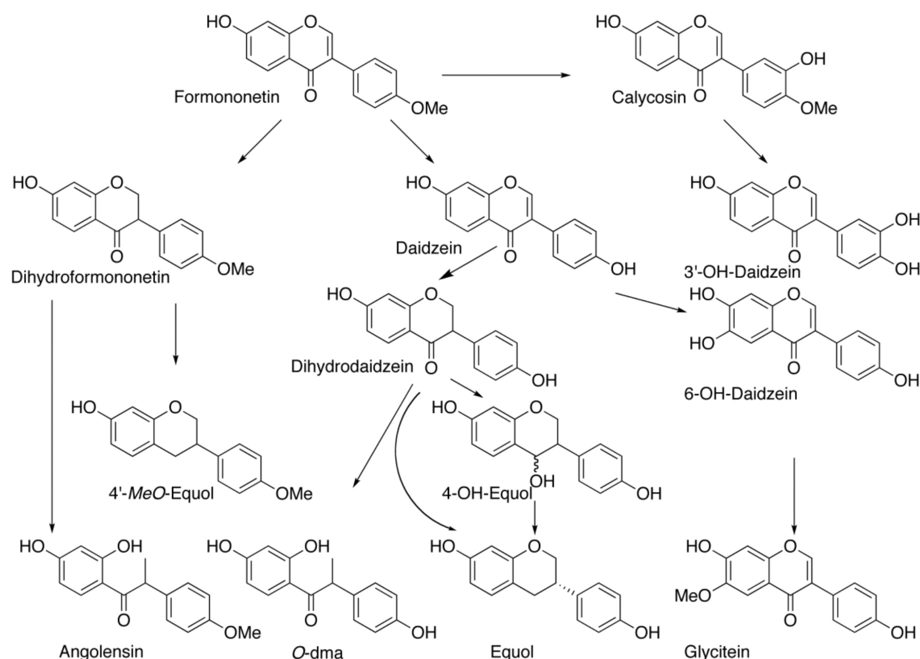
Later on Rüfer et al. studied the in vitro and in vivo metabolism of isoflavone glycitein using similar methods as Kulling [Ruefer et al., 2007]. Aroclor-induced Wistar rat microsomes converted GLY to ten metabolites while human liver microsomes converted GLY to six metabolites. The major metabolites in all microsomal incubations of DA, GEN, and GLY seem to be hydroxylated in the 8-position of the isoflavone ring. Again the identification was based on authentic standards and mass fragmentation.

Incubation of EQ, the main microbial metabolite of DA, with rat and human liver microsomes yielded 11 and six hydroxylated metabolites, respectively. In this case 3'-hydroxy EQ was the predominant metabolite.

1.4.2 PHASE I MICROBIAL METABOLISM

The metabolism of isoflavones intestinal bacteria has been studied both in vitro and in vivo. It has been shown that isoflavone aglycones are subjected to metabolic processes such demethylation and reduction by gut microflora (Scheme 2). Hur et al. reported the biotransformation of FO, BA and, GLY to their corresponding demethylated isoflavones DA, GEN and 6,7,4'-trihydroxyisoflavone, respectively [Hur and Rafii, 2000]. The metabolism of DA occurs via two pathways depending on the microflora of the subjects. Daidzein is first reduced to dihydrodaidzein, DHD. Only about 30 % of the Western people and 50-60% of the Asians are able to convert DHD to EQ. This occurs via the isoflavanol tetrahydrodaidzein. The alternative metabolic route of dihydrodaidzein is the formation of O-demethylangolensin, *O*-DMA. The microbial reduction of GEN proceeds in a similar manner. First GEN is

reduced to dihydrogenistein, which is then transformed to 6’-OH-*O*-DMA [Heinonen et al., 1999, 2003]. It is noteworthy that no isoflavan metabolites for genistein have been identified. This is probably due to the stabilising effect of the hydrogen bond between the keto group and the hydroxy group at 5-position of GEN.



Scheme 2 Metabolism of formonetin in ruminants.

1.4.3 PHASE II METABOLISM

Isoflavonoids are conjugated in liver or within intestinal epithelium with glucuronic acid and sulfate to produce glucuronidines, sulfates and also sulfoglucuronides (Figure 3) and subsequently excreted. In human urine 1-3% of genistein is in free form, 62-64% as monoglucuronide, 13-19% as diglucuronide, 6-12% as sulfoglucuronide, 2-3% as monosulfate and 3-6% as disulfate.

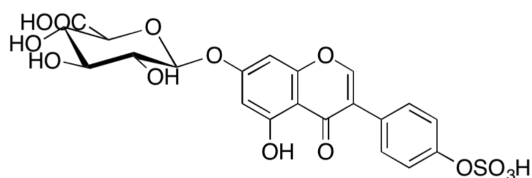


Figure 3 Genistein sulfoglucuronide

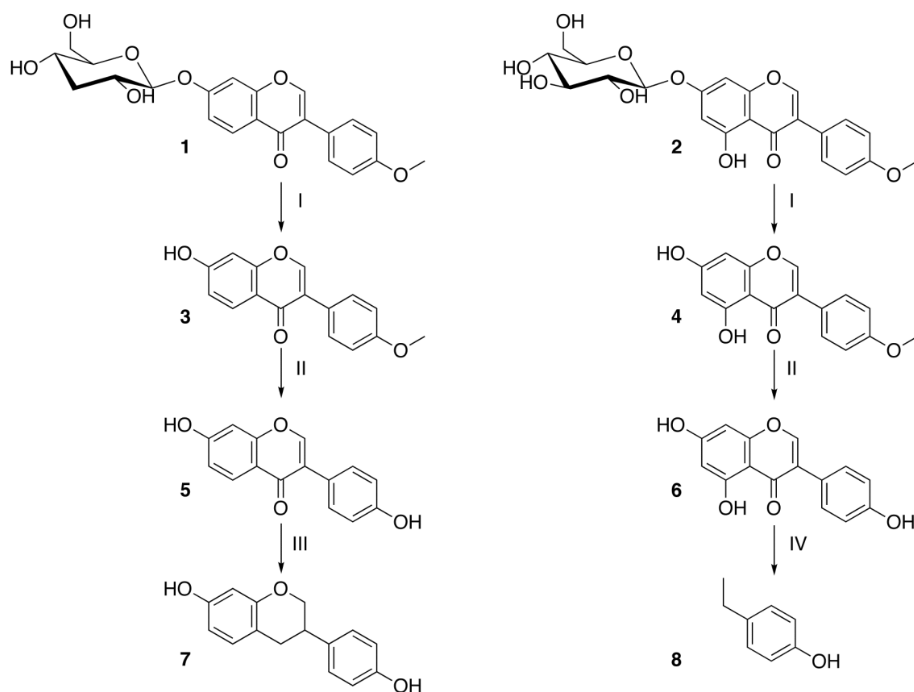
1.4.4 METABOLISM OF RED CLOVER PHYTOESTROGENS IN RUMINANTS

Red clover (*Trifolium pratense L.*) is a leguminous plant, known to contain phytoestrogenic isoflavones. Although it is not used as food, its importance has grown due to various health claims surrounding phytoestrogens. Red clover based supplements for alternative hormone replacement therapy (HRT) has been available for several years. When red clover isoflavone profiles are compared to those of beans such as soy, it is clear that they differ significantly. The main isoflavones of red clover are BA and FO, the methylated derivatives of GEN and DA, respectively. Furthermore, red clover contains several minor isoflavones that are not present in soy (Figure 4).

The isoflavones are present as glycosides in Leguminosae plants. The major metabolic action takes place in the rumen, where isoflavones are hydrolysed and transformed to different metabolites by microorganisms as shown in Scheme 2. Very little if any demethylation and conjugation of formononetin and daidzein occurs in liver microsomes of sheep and cows [Nilsson, 1963, Lundh et al, 1988]. The rumen is the most important site in the gastrointestinal track as regards detoxification of isoflavones, even if conjugation does take place in the rumen, reticulum, omasum, and in small intestines epithelium. The ingested food stays in rumen for some days and rumen micro-organisms are mainly responsible for metabolizing clover isoflavones further [Lundh, 1990, 1995]. The main absorption of the isoflavones to blood plasma probably occurs in the rumen [Cox and Braden, 1974]. Isoflavones and their metabolites circulating in blood plasma are predominantly in the form of glucosiduronates and in this form they are probably biologically inactive [Shutt et al., 1967].

It was first reported by Nilsson that BA is demethylated to genistein in the rumen fluid from sheep and cattle, and two unknown metabolites were also detected in that study [Nilsson, 1961]. In 1962 Nilsson showed how FO is demethylated to DA in the rumen fluid of sheep [Nilsson, 1962]. A few years later Batterham et al. were able to show how intraruminally given BA and genistein were extensively degraded to para-ethyl phenol as a major metabolite [Batterham et al., 1965]. Later Nilsson et al. incubated synthetic tritium labelled BA and FO in rumen fluid in an in vitro study [Nilsson et al., 1967]. BA was degraded to GEN and some unidentified simple phenolics. After 2 h incubation of synthetic FO, the rumen fluid contained FO, DA and EQ.

In their review Cox and Braden concluded that GEN and BA are metabolised almost completely to inactive compounds in the sheep rumen [Cox and Braden, 1974]. BA is demethylated to GEN and via ring cleavage to para-ethyl phenol and organic acids (Scheme 3). They also presented how FO is mainly demethylated to DA and further via hydrogenation and ring fission to EQ. Other possible metabolic conversions of FO were to 4'-O-methyl-equol or angolensin, also one unidentified compound was found.



Scheme 3 Metabolism of FO (**3**) and BA (**4**) according to Cox and Braden [1974].

1.5 ISOFLAVONES IN RED CLOVER

Another aspect of interest of red clover is its use as fodder. In cattle nutrition, forage plants such as grasses and legumes have an essential role. Especially in organic farming, where the use of fertilizers is limited, forage legumes are important due to their capability to bind nitrogen from air. Leguminous plants, clovers, and alfalfa in particular, contain significant amounts of phytoestrogens, isoflavones, and coumestans [Saloniemi, 1995]. In Finland, red clover is the predominant legume cultivated in organic dairy farms. In conventional nonorganic dairy production, however, the use of legumes is scarce, and grass silage based ratios supplemented with barley, oats, and rapeseed meal are the most typical dairy cow ratios during indoor feeding [Vanhatalo, 2006].

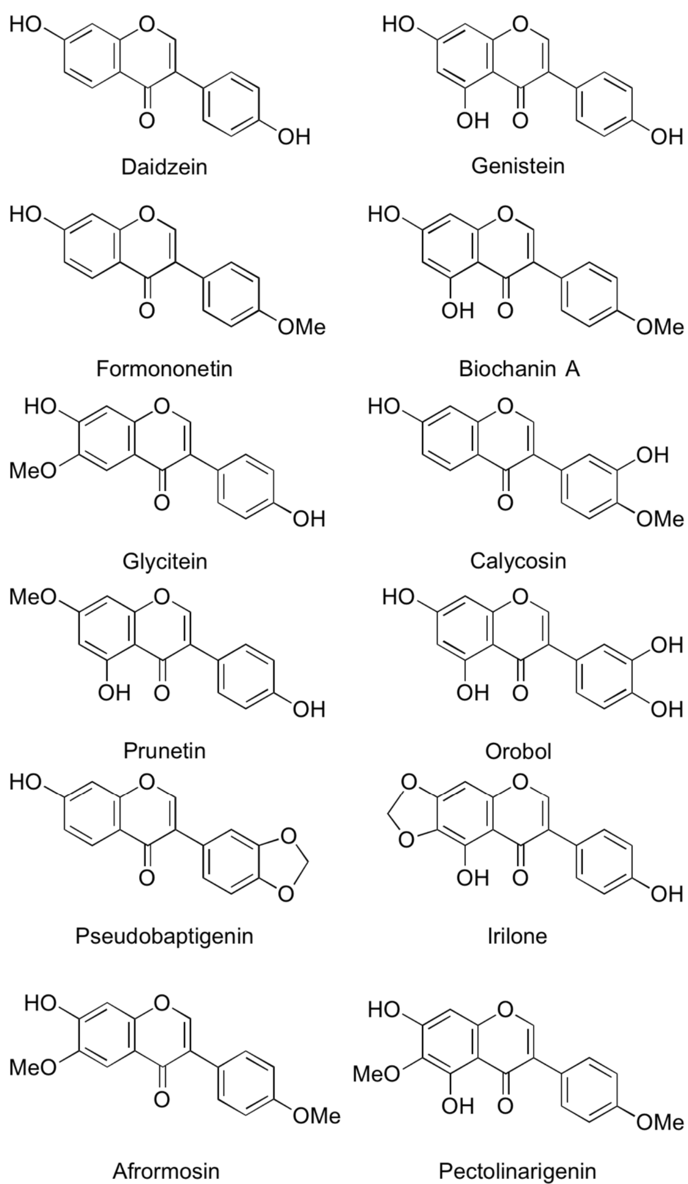


Figure 4 Red clover isoflavones according to LIn et al. [2000].

2 AIMS OF THE STUDY

Isoflavonoid phytoestrogens have raised interest since the early 80's when their beneficial effects on human health was first proposed [Adlercreutz et al., 1982, Axelson et al., 1982]. After that numerous papers have been published regarding the synthesis and analysis of phytoestrogens as well as clinical and epidemiological studies. Relatively little, however, is known about the metabolism of these compounds. Metabolic studies are mainly focused on a few well-known compounds such as equol. It is important to know the metabolism of the compounds since their activity can be greatly altered upon chemical modifications such as reductions or hydroxylations. For example, EQ is estimated to be far more estrogenic than its plant precursors DA or FO [COT, 2003].

Metabolic studies require authentic compounds, that can be used as comparison in the identification of metabolites. Tentative metabolic studies which are based on using similar compounds or interpretation of the physical properties of the compounds, such as their mass fragmentation patterns, offer a good starting point for selecting which compounds are prepared as potential metabolites [Kulling et al., 2001]. Furthermore, the assessment of potential health effects in various in vitro or in vivo studies also requires the use of authentic compounds.

The aims of my work was to prepare potential isoflavonoid metabolites and use the compounds obtained as standards or reference compounds in various studies:

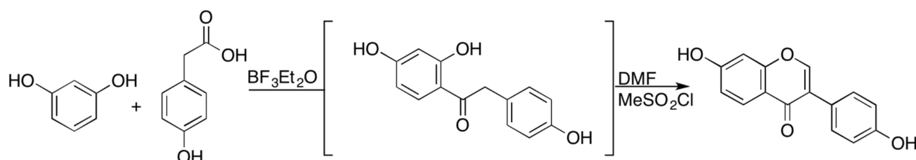
1. Preparation of the parent isoflavone using optimised methods.
2. Preaparation of the metabolites using various synthetic methods e.g. reduction, conjugation, functionalisation.
3. Using the above compounds in chromatographic or clinical studies.

3 RESULTS AND DISCUSSION

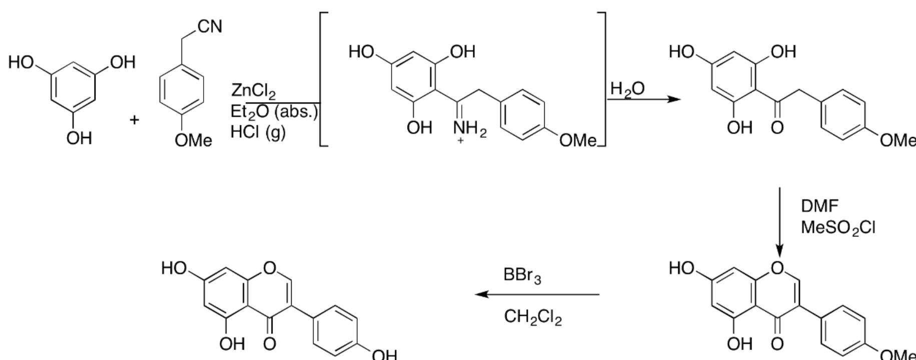
3.1 SYNTHESIS OF ISOFLAVONOIDS

3.1.1 ISOFLAVONES

Total synthesis of isoflavones was performed using two types of Friedel-Crafts acylations (Scheme 4). A one-pot procedure was used for the isoflavones with only one substituent (7-hydroxy) in the A-ring [Wahala and Hase, 1991]. For the isoflavones with multiple substituents in the A-ring a Houben-Hoesch reaction turned out to be preferable (Scheme 5). For the total synthesis, methoxy-substituted isoflavones were generally preferred since hydroxyl-substituted compounds were easily obtained by simple demethylation.



Scheme 4 Synthesis of DA by Friedel-Crafts acylation.

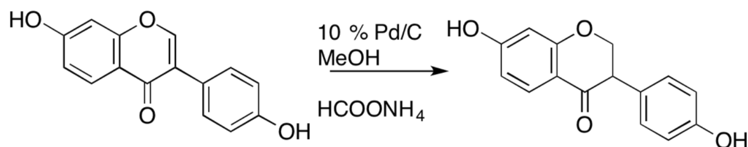


Scheme 5 Synthesis of GEN by Houben-Hoesch reaction.

3.1.2 ISOFLAVANONES

Isoflavanones for this study were prepared by two methods. Hydrogen transfer reaction using ammonium formate and palladium on charcoal (Scheme 6) could be used on all isoflavones [Wahala and Hase, 1989]. Compounds with a hydroxyl group at the 5-position can be also reduced to isoflavanones by a

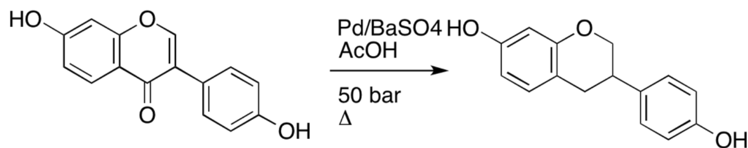
hydrogenation reaction. This is due to the stabilizing effect of hydrogen bond formed between the 5-hydroxyl group and the 4-keto group.



Scheme 6 Synthesis of 7,4'-dihydroxyisoflavanone.

3.1.3 ISOFLAVANS

Isoflavans were synthesized by hydrogenating isoflavones on palladium catalyst supported on charcoal or barium sulfate (Scheme 7). Traditionally isoflavones are reduced at ambient pressure and temperature. This, however, requires high amounts of catalyst and may sometimes lead to unexpected results as reported in the literature [McMurry et al., 1972; Lamberton et al., 1978; Visser and Lane, 1987, Süsse et al., 1992; Chang et al., 1995]. By using high pressure and elevated temperature it was possible to reduce the amount of catalyst needed. Furthermore, the reaction times were also reduced.



Scheme 7 Synthesis of equol.

3.2 MILK STUDIES (I AND II)

In the first study (I), the isoflavonoids formononetin, daidzein, genistein, biochanin A, equol, and *O*-DMA were analysed from commercial cartons of skimmed Finnish milk. Both organic (12 samples) and conventionally produced (4 samples) milk were analysed by HPLC method modified from Franke, Lund, and Antignac et al [Franke et al., 1998a; Antignac et al., 2003; Lund et al., 2004]. Detection was both by UV-DAD and fluorescence detector (FLD). The isoflavones in ruminant feeds are mainly derived from red clover in the Nordic countries. Because forage legumes are able to fix nitrogen they are widely used in organic farming [Taponen et al., 2010].

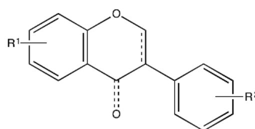
Organic milk contained equol 313 ng/mL-518 ng/mL (mean 411 ng/mL) and only traces of formononetin (mean 4.5 ng/mL) of the compounds analysed. Daidzein was also present in the samples but due to co-eluting impurities we were not able to quantitate the amounts. Conventionally

produced milk contained only equol, but in much lower concentrations (mean 61.6 ng/mL) than in organic milk.

The relatively high concentrations of equol in commercial organic milk prompted us to investigate further the metabolism of isoflavones in cows consuming red clover (II). Milk was collected in a study originally designed to examine the effects of plant oil supplementation of red clover based diets on feed intake and milk production of dairy cows. In short, five Finnish Ayrshire cows were allocated to five experimental diets comprising of red clover silage offered ad libitum and 12 kg/d of concentrates. The silage was prepared from the second growth of pure red clover (variety Ilte). The control concentrate consisted of cereals without oil supplement and the other four concentrates included various plant oil supplements. The concentrates did not contain any phytoestrogen isoflavonoids.

Samples were analysed by the same HPLC method as in the commercial milk study. In this study, however, several other potential metabolites were synthesized and used as standards (Table 1). The synthesized isoflavones were selected based on their occurrence in red clover. All the previously reported red clover isoflavones [Lin et al., 2000; Tsao et al., 2006] were used except for irilone and pratensein. Futhermore, it was also known from human in vitro studies, that polyhydroxylated compounds also could also be produced by microbial transformations [Kulling et al., 2001]. These compounds were used because the reported metabolites of isoflavones are quite similar between humans and animals. Based on this data isoflavones were synthesized and part of them were reduced to isoflavanones and isoflavans.

Table 1. *Compounds analysed.*



Isoflavone			Isoflavanone			Isoflavan		
Compd.	R^1	R^2	Compd.	R^1	R^2	Compd.	R^1	R^2
1a	7-OH	4'-OH	1b	7-OH	4'-OH	1c	7-OH	4'-OH
2a	7-OH	4'-OMe				2c	7-OH	4'-OMe
3a	5,7-OH	4'-OH	3b	5,7-OH	4'-OH	3c	5,7-OH	4'-OH
4a	5,7-OH	4'-OMe						
5a	6-OMe, 7-OH	4'-OH	5b	6-OMe, 7-OH	4'-OH	5c	6-OMe, 7-OH	4'-OH
6a	6,7-OH	4'-OH						
7a	7-OH	3'-OMe, 4'-OH				7c	7-OH	3'-OMe, 4'-OH
8a	5-OH, 7-OMe	4'-OH						
9a	7-OH	3',4'-OCH_2						
10a	5,7-OH	3'-OMe	10b	5,7-OH	3'-OMe	10c	5,7-OH	3'-OMe
11a	5,7-OH	3',4'-OH	11b	5,7-OH	3',4'-OH	11c	5,7-OH	3',4'-OH
12a	7-OH	3'-OMe				12c	7-OH	3'-OMe
13a	7-OH	3'-OH				13c	7-OH	3'-OH
14a	7-OH	2'-OMe				14c	7-OH	2'-OMe
15a	7-OH	2'-OH				15c	7-OH	2'-OH
16a	7-OH	3',4'-OH				16c	7-OH	3',4'-OH

The results (Table 2) show levels of EQ that are much higher than in a study using similar conditions, 918 µg/L vs. 643 µg/L (n) even though the total intake of formononetin was then higher, 75.6 g/d vs. 63.2 g/d (DA 1.8 g/d (x) vs. 7.8 g/d) [Mustonen et al., 2006]. It is clear that most of the equol in milk is derived from formononetin, which is the main isoflavone in red clover. Daidzein is present in much smaller concentrations. Similar results were also reported by Steinshamn et al [2008] who studied the effects of clover-grass silages and concentrate supplementation on the content of phytoestrogens in dairy cow milk. They identified the isoflavones FO, DA, biochanin A, GEN, PR and isoflavan EQ from cow milk but did not study the metabolism further. They found up to 364 µg/L equol in milk when the daily intake of formononetin was 47.0 g/d (daidzein 1.41 g/d). Nielsen et al. investigated the estrogenic activity of bovine milk high or low in equol [Nielsen et al., 2009]. The high equol milk they used was obtained from cows grazing red clover and contained equol 1003 µg/L but the daily intake of isoflavones is not reported. King et al. [1998] found from Australian farm milk equol concentration up to 293 µg/L. Andersen et al. [2009] carried out a grazing experiment to study the concentrations of phytoestrogens in herbage including red clover and in milk. Concentrations of FO and BA in herbage were higher than other studies 11,4 g/kg and 8,89 g/kg, respectively. EQ concentration in milk was 355 µg/l. Antignac et al. [2004] studied isoflavonoid concentrations in French organic and conventional milk. The average EQ content of organic samples was 191 µg/L. The high level of EQ in this study is more than likely affected by the relatively high intake of DA compared to other studies.

Table 2. *Isoflavonoid concentrations in silage, milk, and plasma according to various studies A: [Mustonen et al., 2006], B: [King et al., 1998], C: [Steinshamn et al., 2008], D: [Andersen et al, 2009], E: [Nielsen et al., 2009], F: [Antignac et al., 2004].*

	I		II	A	B	C	D	E	F	
Concentration in silage, g/kg DM										
Daidzein	n/a		0,624	0,16	n/a	0,091	*	n/a	n/a	
Formononetin	n/a		5,007	6,47	n/a	3,039	11,4	n/a	n/a	
Genistein	n/a		0,609	0,5	n/a	0,073	*	n/a	n/a	
Biochanin A	n/a		2,957	4,65	n/a	0,482	8,89	n/a	n/a	
Prunetin	n/a		0,772	n/a	n/a	0,066	n/a	n/a	n/a	
Glycitein	n/a		nd	n/a	n/a	n/a	0,91	n/a	n/a	
Intake of phytoestrogens, g/d										
Daidzein	n/a		7,8	1,83	n/a	1,0		n/a	n/a	
Formononetin	n/a		63,2	75,6	n/a	33,7		n/a	n/a	
Genistein	n/a		7,7	n/a	n/a	2,2		n/a	n/a	
Biochanin A	n/a		37,7	n/a	n/a	22,7		n/a	n/a	
Prunetin	n/a		9,7	n/a	n/a	2,7		n/a	n/a	
Concentration in milk, µg/l	OM	CM							OM	CM
Equol	411	61,6	918	643	293	364,0	355,4	1000,3	191	36,4
3',7-Dihydroxyisoflavan	n/a	n/a	800	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Formononetin	4,5	nd	2,4	nd	n/a	6,96	6,28	20,4	3,4	0,3
Dihydrodaidzein	n/a	n/a	61,4	n/a	n/a	n/a	n/a	n/a	n/a	n/a
O-DMA	nd	nd	2,7	nd	n/a	n/a	n/a	n/a	n/a	n/a
Daidzein	*	*	*	nd	5	7,71	1,14	5,7	3,9	1,0
Biochanin A	nd	nd	nd	nd	n/a	2,13	0,55	nd	1,8	0,3
Genistein	nd	nd	nd	nd	2	3,02	*	n/a	2,1	0,5
Prunetin	n/a	n/a	nd	n/a	n/a	1,01	n/a	n/a	n/a	n/a
Glycitein	n/a	n/a	nd	n/a	n/a	n/a	n/a	10,2	n/a	n/a
Secretion in milk, mg/d										
Equol	n/a		28,6	19,1	n/a	n/a	n/a	n/a	n/a	
3',7-Dihydroxyisoflavan	n/a		25,8	n/a	n/a	n/a	n/a	n/a	n/a	
Concentration in plasma, mg/l										
Equol	n/a		n/a	8,39	n/a	n/a	n/a	n/a	n/a	
O-DMA	n/a		n/a	0,32	n/a	n/a	n/a	n/a	n/a	
Formononetin	n/a		n/a	0,035	n/a	n/a	n/a	n/a	n/a	

OM: Organic commercial milk.
CM: Conventionally produced commercial milk.
*: Detected but not measured; **: Grazing study, no silage used.

Based on the various studies it is clear that equol concentrations in milk may vary significantly. The main reason for this is the differences in cow feeds.

However, EQ concentrations in milk do not always correlate directly with the isoflavone amounts in the feed when studies are compared with each other. This suggests that there are also major differences between the individual animals.

One of the important results in our study was the identification of 3',7-dihydroxyisoflavan as another major isoflavone metabolite along with equol. Although it has been identified in cow's milk before [Bannwart et al., 1988], it has never been quantified and so its importance has been overlooked. EQ is considered as probably the most important phytoestrogen and 3',7-dihydroxyisoflavan is structurally closely related to it. The only difference is the position of the hydroxyl group of the B-ring at the neighbouring carbon. Thus it can be postulated that 3',7-dihydroxyisoflavan shares similar properties and has to be taken into consideration when the estrogenicity is evaluated. In retrospect it was also noticed from the chromatogram that the commercial milk samples from the first study (I) also contained a peak at the same retention time than the peak corresponding 3',7-dihydroxyisoflavan found in the red clover study (II). The presence of 3',7-dihydroxyisoflavan in the first studyhas not been confirmed but it is likely that metabolites are the same.

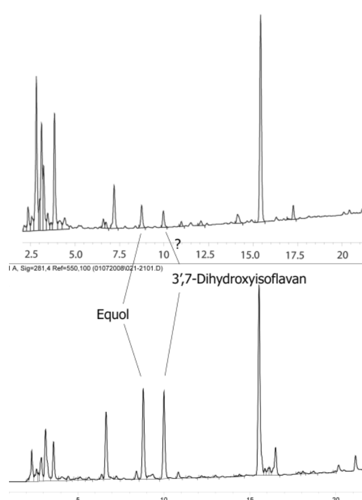
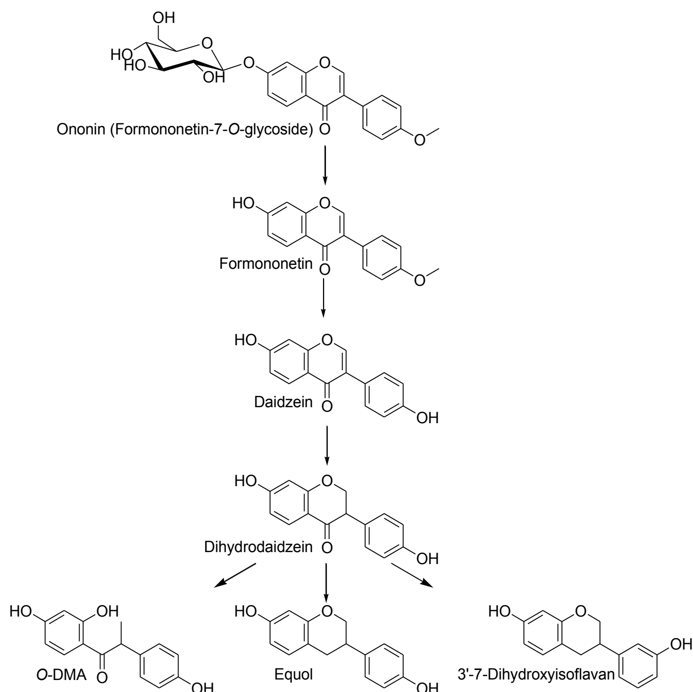


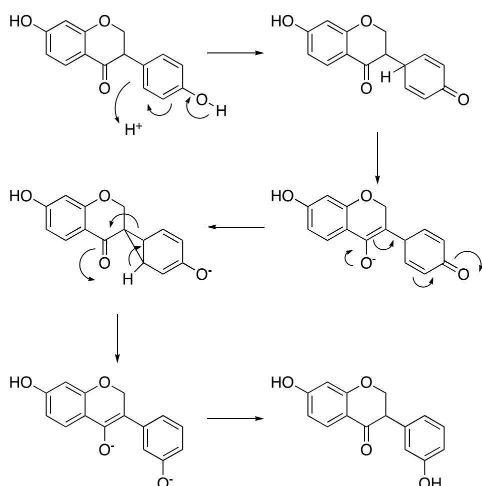
Figure 5 Comparison of HPLC chromatograms of organic commercial milk study (I, above) and milk form red clover feeding study (II, below).

The red clover feeding study was the first where dihydrodaidzein and *O*-DMA were reported in milk. The presence of these compounds makes it possible to propose a metabolic pathway of red clover isoflavones FO, DA and their glycosides. Unconjugated isoflavones are first reduced to isoflavanone dihydrodaidzein followed by further reduction either via ring opening to produce *O*-DMA or by the route leading to the isoflavan EQ (Scheme 8). The

high concentration of 3',7-dihydroxyisoflavan (Figure 5) suggests that it is forms as a product of metabolism of formononetin and daidzein, but its formation requires the migration of hydroxyl group from 4'-position to neighbouring carbon atom. Such a migration has not been presented before and it is unclear at which step this occurs. Alternatively, a spirocyclic intermediate may be involved to explain the apparent migration (Scheme 9) or it may be hydroxylation/dehydroxylation.



Scheme 8 Proposed metabolic pathway of ononin, formononetin and daidzein.



Scheme 9 Proposed spirocyclic intermediate for hydroxyl migration.

3.3 ISOFLAVONE FATTY ACID ESTERS (III)

Oxidation of low-density lipoprotein (LDL) plays an important role in the advancement of atherosclerosis. It has been shown that antioxidant isoflavones such as genistein protect lipids from oxidation in several lipid-aqueous systems *in vitro*. [Kapiotis et al., 1997; Ruiz-Larrea et al., 1997]. Also, dietary administration of phytoestrogens inhibits LDL oxidation in human ex vivo [Tikkanen et al., 1998]. It has been shown that human plasma can convert genistein into lipophilic derivatives, part of which have been identified as genistein fatty acid esters that accumulated in lipoproteins [Kaamanen et al., 2003]. Furthermore, the fatty acid esters of both isoflavones as well as estradiol seem to have remarkably stronger antioxidant potential on LDL particles compared to their non-esterified forms [Shwaery et al., 1998]. The free form also incorporates only to a small extent in LDL, whereas esterification enhances this incorporation [Meng et al., 1999]. Isoflavones are possibly modified in humans similarly to the endogenous steroid hormone fatty acid esters, which circulate in blood bound to lipoproteins.

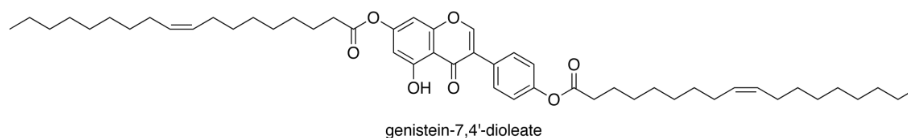


Figure 6 Genistein-7,4'-dioleate.

To study the clinical effects of isoflavone fatty acid esters, GEN and DA were esterified with stearoyl and oleyl chlorides (III). Both 7- and 4'-fatty acid

monoesters and 7,4’-diesters were produced [Lewis et al., 2000]. It has been shown that in GEN and DA, the 7-OH exhibits a hundredfold acidity compared to the 4’-hydroxyl group. This property can be utilised in selective mono O-acylations of the isoflavones. Using one equivalent of potassium t-butoxide forms the 7-phenolate, which can then react with acyl chloride yielding 7-mono fatty acid ester. 2,2-3,3 equivalents of base were used for forming the 4’,7-diphenolate. The greater nucleophilicity of the 4’-phenolate compared to 7-phenolate results in selective acylation at 4’-position when one equivalent of acyl chloride is used. Diesters are produced by using two equivalents of acyl chloride. The 5-hydroxyl group of GEN is stabilised by hydrogen bonding with the keto-group at the 4-position preventing the formation of triesters under 30 °C. It has been demonstrated that oleic acid esters of daidzein and genistein are incorporated into LDL with an efficiency comparable to α-tocopherol [Meng et al., 1999]. Stearic acid esters of genistein are less effective as are also free isoflavones. The ineffective incorporation of unesterified isoflavones is explained by their relatively low lipid solubility. The difference between stearic and oleic esters is probably due to their conformation. Molecular modelling suggests that in genistein-7,4’-dioleate, both fatty acid chains are folded around genistein. (Figure 7) Thus the molecule could be more easily incorporated into LDL.

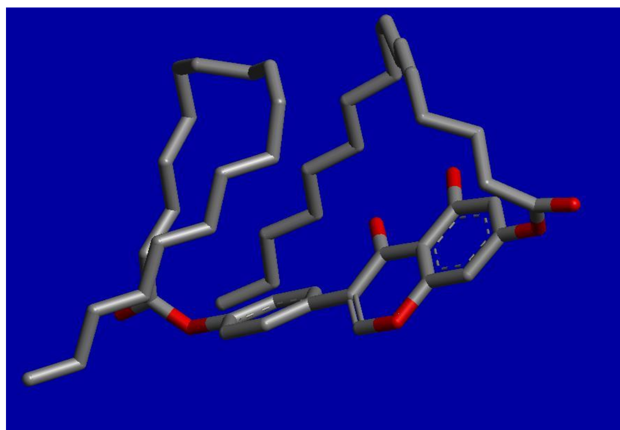


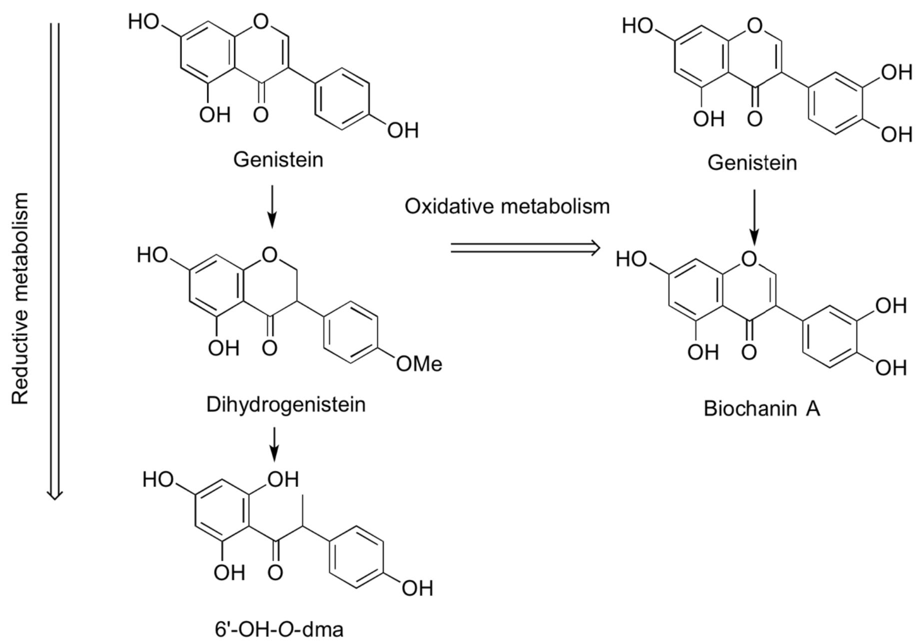
Figure 7 Minimised structure of genistein-7,4’-dioleate .

GEN and genistein 4’,7-dioleate were administered to adult rhesus monkeys orally or subcutaneously to determine genistein fatty acid esters in plasma (III). Tritium labelled genistein ([^{3}H]genistein, SibTech, Inc., USA, specific activity 20–25 and >30 Ci/mmol) was used as a starting material to produce labelled genistein 4’,7-dioleate with a procedure described previously. It was used as an internal standard in a method developed for the quantitative determination of plasma GEN and GEN fatty acid concentrations. The genistein fatty acid esters were detected from non-human primate plasma

after subcutaneous injection of genistein dioleate. The results suggest that it may be possible to introduce genistein ester molecules into plasma by parenteral but not oral administration. More recently a Chinese study reported the effects of isoflavone fatty acid esters on mice body weight and hypolipidemic activities [Xiang et al, 2010]. Formononetin-7-oleate was found to be the most potent compound.

3.4 METABOLISM OF SOY ISOFLAVONES IN HUMAN SUBJECTS (IV)

Metabolism of the soy isoflavones DA, GEN, and GLY in human subjects was studied using authentic reference compounds and by the interpretation of mass spectra (IV). In the study six volunteers included three soy bars per day into their normal diet. Daily urine samples were collected and analysed as trimethylsilyl derivatives using GC-MS. The metabolites, for which authentic reference compounds were not available, were identified by the interpretation of mass spectra. The study focused on phase I metabolism of isoflavones and several new metabolites were identified (Scheme 10). The presence of previouosly reported reduced metabolites of DA and GEN, dihydrodaidzein, EQ, *O*-DMA and cis-4-OH-equol, dihyrogenistein, and 6'-OH-*O*-DMA was confirmed. For the first time, the identification of reduced metabolites of glycitein, dihydroglycitein, 5'-OMe-*O*-DMA and 6-OMe-equol was reported in humans. The results suggest that also oxidative metabolic pathways exist for isoflavone metabolites, by identifying the hydroxylated metabolites of DA and GEN. Additionally, liver metabolites that undergo enterohepatic circulation may be subjected to reductive reactions by gut microflora.



Scheme 10 Proposed metabolic pathway of genistein (IV).

4 CONCLUSIONS

Isoflavones undergo several metabolic reactions in cows. We found that EQ is the main metabolite as also shown in previous studies. Another major metabolite we found was 3',7-dihydroxyisoflavan, a compound which was previously identified but has never been quantified. It was surprising to find that its concentration was similar to that of EQ. It is also worth mentioning that the silage which was fed to cows did not contain any natural isoflavone precursor for the 3',7-dihydroxyisoflavan corresponding to those which are commonly thought to be precursors of EQ, namely DA and FO. This means that 3',7-dihydroxyisoflavan has to undergo more complex metabolism than previously proposed.

The presence of isoflavonoids in commericial milk depends on the fodder that the cows are fed. Our study shows that relatively high levels of equol are present in Finnish organic milk whereas conventionally produced milk contains much smaller amounts. This is probably due to the use of red clover in organic farming to fix nitrogen in the fields. Red clover contains considerable amounts of FO, which is converted to EQ in the alimentary track of ruminants. Bovine milk could therefore be seen as a source of EQ, especially among persons who are not capable of converting isoflavones, derived from plants such as soy, to EQ.

The parenteral administration of fatty acid esters of genistein was explored by subcutaneous injection to adult female rhesus monkeys. We were able to show that it may be possible to introduce intact genistein molecules into plasma by parenteral administration. These molecules may have antiatherogenic properties such as the protection of LDL particles from oxidation at physiologically relevant concentrations.

Phase I metabolism of isoflavones was studied in human urine, collected after soy supplementation. Several new metabolites were identified using authentic reference compounds or by the interpretation of mass spectra. The results suggest that oxidative metabolic pathways also exist for isoflavone metabolites formed by gut microflora. Also liver metabolites that undergo enterohepatic circulation may be subjected to reductive reactions although to a lesser extent. In conclusion, the metabolism of isoflavones in humans is diverse leading to a wide range of metabolites.

The metabolic studies relating to this work have revealed many new metabolites as well as confirmed previous studies. It has been shown, that to fully understand the relevance of the phytoestrogens in food, requires also an understanding of what is their fate after consumption. It can be seen that individual variations, especially in humans, play an important part. Futhermore, the food produced from animals consuming isoflavones can be a source of potentially beneficial isoflavone metabolites for those people who are

unable to produce them by themselves. It is, however, clear that further studies are needed in order to reveal the true significance of the compounds.

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