The effect of processing and formulation on the bioavailability of isoflavones from red clover

Dorthe Møller Sørensen
Title: The effect of processing and formulation on the bioavailability of isoflavones from red clover

Danish title: Effekten af forarbejdning og formulering på biotilgængeligheden af isoflavoner fra rødkløver

Project period:
4th February 2014 – 4th May 2015
3rd and 4th semester MSc

Author: Dorthe Møller Sørensen
Student number: 201210044

Supervisor: Per Bendix Jeppesen
Co-supervisor: Max Norman Tandrup Lambert

M.Sc. Molecular nutrition and food technology
Aarhus University – Department of food science
Preface

This master thesis has been carried out in the 3rd and 4th semester of the MSc programme in Molecular Nutrition and Food Technology, Aarhus University, Denmark. The project was conducted under supervision of Associate Professor PhD, Per Bendix Jeppesen with the help from PhD stud., M.Sc. Max Norman Tandrup Lambert.

The project is presented as a literature review and a scientific article based on experimental work carried out at the Diabetes Research Laboratory, the Department of Endocrinology, MEA, Aarhus University Hospital. The project is part of a larger research project on osteoporosis in menopausal women formulated by Associate Professor PhD, Per Bendix Jeppesen.

The review article titled “Bioavailability and physiological effects of isoflavones – a review” will be submitted to the journal “Journal of natural products”.

Due to problems with the highly sensitive and advanced analytical equipment necessary for analyses of plasma samples, it has only been possible to analyse a minor portion of the plasma samples from the clinical study within the timeframe of this project. The scientific article titled “Bioavailability of isoflavones from fermented and unfermented red clover formulations” is therefore based on results from approximately 10% of the samples. The remaining samples will be analysed and results incorporated in the article at a later time.

Acknowledgements

I would like to thank my supervisor Associate Professor PhD, Per Bendix Jeppesen for supervision during the project period, and PhD stud, M.Sc. Max Norman Tandrup Lambert for practical help during the study, sharing of theoretical knowledge and proof reading.

A big thank you goes to laboratory technicians Lene Trudsø and Eva Mølgård Jensen and M.Sc Catrine Thybo for all their practical assistance and huge support during the study. I wish to thank PhD stud. Joan Bach Nielsen and PhD stud. Thomas Nordstrøm Kjær for their assistance with blood sampling during the study, as well as the Biochemical Department at Aarhus University Hospital THG. A thank you also goes to the participants for making the study possible and being good sports.

I’d like to thank Lars Jørgensen, DBlab for analysis of red clover formulations, Hans-Christian Vollstedt and Henrik Jørgensen, Natur-Drogeriet A/S for production of capsules and tablets and Xavier Fretté, Rime Bahij El-Houri and Flemming Nielsen, SDU for analysis of plasma samples.

Moreover, I want to thank Michael Mohr Jensen, Herrens Mark, for producing and providing red clover extract as well as funding for the project.
Bioavailability and physiological effects of isoflavones – a review

Author name: Dorthe Møller Sørensen
Address: Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Tage-Hansens Gade 2, DK-8000 Aarhus C, Denmark
E-mail: dorthe_ms@yahoo.dk

Abstract

Isoflavones are phytoestrogens found in plants that resemble estrogen in chemical structure, and bind to estrogen receptors with a preference for estrogen receptor β (ERβ). They can act as estrogen agonists or antagonists and they may exert tissue specific effects. Soy and red clover (RC) are rich sources of isoflavones, which has led to the prospect of soy and RC being alternatives to conventional hormone replacement therapy (HRT). Following ingestion the isoflavones are metabolised by enzymes, absorbed, conjugated in the liver, circulated in plasma and finally excreted in urine. Bioavailability is important for the biological activity of isoflavones and many factors affect isoflavone absorption including isoflavone source and dose, food matrix and individual metabolism. Accumulating evidence suggests that isoflavones may potentially confer health benefits to many diseases including menopausal symptoms, osteoporosis, hormone-related cancers and cardiovascular diseases (CVD). These results are consistent with epidemiological evidence showing lower incidences of cancer and CVD in populations consuming diets high in soy. Although a few concerns have been raised about soy intake being harmful, especially to breast cancer patients, the intake of isoflavones is generally evaluated as safe. The aim of this review is to summarize data on bioavailability and health effects of isoflavones.

1 Introduction

The menopause results in a reduction in production of endogenous estrogen, and a hypoestrogenic state that can cause menopausal symptoms. Symptoms include mood and behavioural changes, hot flushes, sleep disturbance, vaginal dryness and more. Lack of estrogen production is also associated with an increased risk of suffering from diseases like osteoporosis and cardiovascular disease (CVD). The risk of cardiovascular morbidity and mortality is lower in premenopausal women compared to men of the same age, but the risk increases as women reach menopause [109]. Due to an increased rate of bone loss during menopause, postmenopausal women have an increased risk of developing osteoporosis in the long run [3]. Currently hormone replacement treatment (HRT) is available, however usage is associated with side effects of which most concerning is an increased risk of cancer. Alternatives like phytoestrogens from plants are becoming increasingly popular as they are thought to proffer the same beneficial effects of HRT without proffering side effects.
Asians have lower incidences of CVD, prostate and breast cancer compared to western populations, and Asian women experience fewer menopausal symptoms [54,151]. Hendrich et al. have shown that the plasma level of daidzein was higher in Japanese women than in Asian women who had immigrated to Hawaii, and the incidence of breast cancer was lower for Japanese women [38]. Asian migrants who maintain their usual diet do not have increased risk of coronary heart disease and hormone related cancer types, compared to migrants who change to a western style diet [54]. This suggests that dietary and environmental factors have an impact apart from racial differences, and that a higher intake of phytoestrogens from soy products results in a lower risk of developing diseases associated with estrogen deficiency [47,101,134,151].

There is growing evidence that isoflavones have beneficial effects on menopausal symptoms and hormone-dependent disease, but inconsistencies are present in this field. This paper introduces chemistry, metabolism and bioavailability of isoflavones, and summarizes results from studies on the physiological effects of isoflavones (incl. osteoporosis, cancer, diabetes, menopausal symptoms and CVD).

2 Estrogen and HRT

Estrogens are the main sex hormone in females, and are C18 steroids derived from androgens, which are synthesized from cholesterol. Several types of estrogen exist, and 17 β-estradiol (E2) is the most potent form followed by estrone (E1) and estriol (E3) [36]. The latter two require conversion to E2 locally in order to attain full estrogenic activity [83]. E2 is primarily formed in the granulosa cells of the ovaries, and E1 and E3 are mainly formed from E2 in the liver [36]. Aromatase enzymes catalyse the final step in estrogen formation and are mostly expressed in ovarian granulosa cells in premenopausal women [36,83]. In postmenopausal women the main sites of aromatase expression are extragonadal sites like adipose tissue and skin [83].

Estrogen action is mostly mediated through estrogen receptors (ERs) which function as ligand-activated transcription factors [83]. Binding of a ligand to the receptors results in conformational changes, which leads to changes in the rate of transcription of estrogen-regulated genes. In the human body, there are two different types of ERs; ERα and ERβ, which are different in structure and biological function, and ligands have different binding affinities for the two receptors [36,80,83]. Endogenous estrogen binds mainly to ERα [140]. The two receptors are expressed differently in different tissues, where ERα is found mainly in breast and ovarian tissue and ERβ is found in brain, bone and bladder tissues [36,140].

Apart from these two ERs that are both found inside the cells, recent research has found G-protein coupled receptors in cell membranes, which are activated by estrogen. The mechanisms and involvement of these receptors in physiological processes are still being researched [70].
In breast tissue, estrogen stimulates growth of the ductal epithelium and connective tissue. Estrogen however can impact negatively on this tissue, as it stimulates the growth of breast cancer cells [36]. Estrogens affect the cardiovascular system because they are natural vasoprotective agents. They increase the formation and release of nitric oxide (NO) and prostacyclin in endothelial cells as well as reduce vascular smooth-muscle tone. Controversy exists about whether estrogen treatment can prevent atherosclerosis in postmenopausal women as epidemiological and intervention studies show contradicting results [36]. Estrogen also affects bones as osteoclasts and osteoblasts contain ERs. In osteoblasts, estrogen stimulates the secretion of the anabolic growth factor IGF-1, and inhibits the secretion of cytokines, IL-1, TNF and IL-6 that are involved in bone resorption. Estrogen also inhibits the function of osteoclasts by stimulating the synthesis and secretion of osteoprotegrin (OPG). Estrogen is thereby involved in reducing bone resorption and maintaining bone health [36,86].

When women reach the perimenopausal stage, ovarian production of E2 decreases, which results in decreasing plasma levels of E2. The E2 which is present is formed in extragonadal tissues and the amounts that are synthesized increase with body weight [36]. Estrogen deficiency in postmenopausal women is partly responsible for decreases in bone mass and increased risk of fracture over time, as estrogen deficiency leads to increased bone resorption and decreased formation of new bone tissue. Lack of activation of the ERs in bone tissue, leads to a decline in secretion of the growth factor IGF-1 and OPG, stimulating bone remodelling and inhibiting osteoclast function respectively [36,88,140].

HRT is a treatment used for menopausal women to relieve menopausal symptoms and/or decrease the risk of hormone-dependant diseases. The treatment includes estrogen sometimes combined with a progestin, which is a synthetic progesterone that ought to counteract the proliferative action of estrogen in the endometrium [22]. HRT is associated with an increased risk of breast cancer in postmenopausal women, and the risk increases with duration of use [11,24]. The Women’s Health Initiative (WHI) study, a large randomized controlled study, found that HRT use was associated with a 26% increased risk of developing breast cancer [102]. A recent meta-analysis of 52 epidemiological studies revealed that current or recent users of HRT had a 37% increased risk of developing ovarian cancer [23]. There are contradicting results regarding the effect of combining estrogen with progestin. It is unclear whether progestin exerts proliferative or antiproliferative effect on the human breast, as study results depend on the structure of progestin and the chosen tissue [107]. However, there are several large observational studies that indicate a further increase in cancer risk when using progestin, including The Nurses’ Health study reporting a yearly increase in cancer risk of 3.3% when using estrogen alone compared to 9% for estrogen plus a progestin [22]. In The Million Women study including 828,923 postmenopausal women they found a relative risk (RR) of 1.66 for developing breast cancer in current users of HRT compared with never users. The risk was greater for users of estrogen-progestin preparations than for estrogen-only preparations (RR: 2.0 and 1.3 respectively) [11].

Estrogen has been shown to have vasculoprotective effects including beneficial effects on the lipid profile and NO production. In The Nurses’ Health study the risk of coronary events was reduced by 45% in users of HRT [35]. There is however also evidence of the opposite including the WHI study.
which was ended prematurely because of an increased risk of coronary events [102]. These contradictions could be attributable to biases in observational studies, but there might also be a timing effect where the influence of HRT on the risk of CVD is associated with the timing of initiation of HRT (age and time since menopause) [41].

Selective estrogen receptor modulators (SERM) are nonsteroidal compounds that mimic the effect of estrogen in some tissues, but have an antagonistic effect in other tissues [36]. This type of pharmaceuticals is used for treatment of diseases related to hypoestrogenic states including osteoporosis, CVD and cancer [9,90]. The combination of agonistic and antagonistic effects makes SERMs useful for treatment of symptoms of postmenopausal women. The estrogenic effect on bones and brain is desired to maintain bone strength and brain function, and agonistic effects on breast and endometrial tissue are avoided [36].

Isoflavones are considered to be natural SERMs, even though their mechanism of action differs from the synthetically manufactured SERMs [9]. They bind to ERs and are tissue specific in that they have preferential and stronger binding to ERβ compared to ERα [57]. The different isoflavones have varying binding affinities to ERs [90,108]. Their preferred binding to ERβ results in a protective effect against breast cancer as it inhibits mammary cell growth, which is an advantage compared to HRT [90].

3 Isoflavone sources and structures

Secondary metabolites are organic compounds found in plants. Unlike the primary metabolites, they function as part of a plant's defense system. The secondary metabolites are generally divided into three main groups: terpenes, phenolics and nitrogen-containing compounds [13,127]. The phenolic group contains flavonoids that are a type of phytoestrogen, which are nonsteroidal compounds that have structural similarities to estrogens (17-β-estradiol) and can exert estrogenic effects in the body by binding to ERs. Flavonoids are the main type of phytoestrogens found in the western diet, and are a compelling group of compounds from a nutritional and health perspective. Isoflavones which are found in soy and red clover (RC) are part of this group of compounds [112,130,134].

Soy and RC (Trifolium pratense L.), both from the Leguminosae family, are abundant sources of isoflavones [148]. Soy has been part of the traditional Asian diet for centuries, and it has been known since 1931 that soybeans contain high amounts of isoflavones [1]. Soy is to date the most studied source of phytoestrogens. RC is used in agriculture as it improves soil quality by fixing nitrogen. In the 1940s the estrogenic effects of RC isoflavones were first documented, where a decrease in fertility of grazing sheep on RC fields was found; later experiments have shown similar effects in cattle [9].

The main isoflavones found in soy are genistein, daidzein and glycitein [72]. In RC the main isoflavones are formononetin and biochanin A, which is a unique feature of RC. Genistein and
daidzein are the demethylated forms of biochanin A and formononetin respectively, and are found in smaller amounts in RC (Figure 1) [148].

 Isoflavones can exist as aglycones or in a conjugated form as glycosides. In soy and RC the isoflavones are found in a complex mixture of aglycones and the glycoside conjugates β-, malonyl- and acetyl glycosides [15,91]. The malonyl- and acetyl glycosides are less stable than the β-glycosides and readily convert into β-glycosides upon exposure to heat or other processing steps [112]. The β-glycosides are predominant in soy, whereas in RC the isoflavones are mainly found as malonyl glycosides [148,156].

The concentration and composition of the isoflavones in plants depends on factors like plant cultivar, environmental conditions and industrial processing [112]. The pattern of conjugation of the isoflavones affects the ease with which the glycosides are hydrolysed or degraded by bacteria and thereby the bioavailability of the isoflavones [53]. Processing of isoflavone containing foods can alter the total content of isoflavones as well as the ratio between glycosides and aglycones. Generally processing increases hydrolysis and thereby the content of aglycones, and in fermentation processes the glucose moiety is removed and the level of aglycones is increased [74,91]. The aglycones are heat stable, which means that it is mainly the conjugation pattern that is affected by heating processes and not the total isoflavone content [112].

Figure 1: Chemical structure of main isoflavones in soy and red clover and daidzein metabolites.
4 Absorption and metabolism of isoflavones

The gut microflora of an individual influences the bioavailability of isoflavones from the diet. The composition of the gut microflora varies between individuals, and is established early in life (age 0-3 years). It is considered to be relatively stable but dietary changes can affect the composition and activity of colonic microbiota [110].

The glycoside form, in which the isoflavones are mainly found in their natural state, is not highly bioavailable for humans, and a conversion is needed before the isoflavones are absorbed [111,115]. The reason that active transport isn’t possible is thought to be due to their high hydrophilicity and molecular weight [47]. The first step of isoflavone metabolism is the hydrolysis of glycosides into their aglycone form. This hydrolysis is highly efficient and is carried out by glycoside hydrolases along the entire length of the intestinal tract [15,91,101,114]. The intestinal bacteria are also responsible for the demethylation of methylated forms of isoflavones, and less than 5% remain intact [9,111]. The glycoside hydrolases are either endogenously present in the small intestine, eg. lactase phlorizin hydrolase (LPH), or from bacteria colonizing the small and large intestine (β-glucosidases) [85,101].

![Image](image.png)

**Figure 2: Gut microfloral metabolism and distribution of isoflavones.**

Aglycones are absorbed directly or further metabolised into other metabolites [15]. It is believed that the direct absorption of aglycones takes place by non-ionic passive diffusion in the jejunum (Figure...
When ingesting isoflavones there is an increase in plasma concentrations of isoflavones 1-2 hours after ingestion. This reflects direct absorption of the aglycones and is more pronounced when ingesting aglycone rich formulations [47,53,89]. When ingesting glycoside-rich formulations the main peak in most cases appears after 5-8 hours which is due to the hydrolytic cleavage and absorption taking place in the lower part of the small intestine and in the colon, as well as some enterohepatic recycling of conjugates [85,89]. In these cases there might also be a small peak early after ingestion, which is due to some of the hydrolytic cleavage and absorption taking place in the small intestine, and direct absorption of the minor proportion of aglycones present in the formulation [89].

In the second step of isoflavone metabolism the aglycones are either absorbed by the liver to undergo first pass hepatic conjugation, or metabolised further in the large intestine [134]. After absorption in the liver the aglycones are either released in their active form to the blood stream, or undergo conjugation; mostly into glucuronides and a small proportion as sulfates [115,134]. Genistein and daidzein both have two conjugation sites and can therefore be found as mono- or di- glucuronides or sulfates, as well as mixed conjugates with one sulfated and one glucuronidated site [119]. Isoflavones are found in blood either in the aglycone form or as conjugates, and generally low concentrations of aglycones are found in blood as the first pass enterohepatic conjugation is efficient [111,153]. The conjugation is carried out by UDP-glucuronyltransferase isozymes, and they have both tissue and substrate specificity [115]. A study on ewes revealed that in tissues the isoflavones are mainly found as glucuronides, and that there are large variations in concentrations between tissues where the highest concentrations were found in kidney and liver [137].

The formed glucuronides enter the systemic circulation and are transported to target cells and tissues where they exert their biological activity. Some of the isoflavones are excreted in the bile, and intestinal glucuronidases can hydrolyse them making them available for re-absorption and further enterohepatic recycling or for metabolism. The conjugation helps retain the isoflavones in the body within the enterohepatic circulation, and prolongs their pharmacological activity [111,134]. The extent of biological activity of the conjugated isoflavones is at present not fully clarified [153]. Studies have shown that the conjugates might have biological activity through direct actions or by serving as immediate sources of aglycones in target tissues [119]. In an animal study using quercetin it was shown that quercetin aglycones are found in tissues but not in plasma where it is mainly found as glucuronides. β-glucuronidase activity was also found in lung, liver, kidney and muscle tissue and this suggests that the active aglycones are formed at target tissues [12]. The same mechanisms might apply for isoflavones. The glucuronides are water-soluble and are eventually excreted in the urine [151]. It has been shown that the conjugate patterns in urine and plasma are different, which suggests that further metabolism takes place during renal clearance. Double conjugated metabolites are the main conjugates found in plasma, whereas in urine the monoglucuronides predominate. This suggests that the highly polar double conjugates cannot readily undergo renal excretion, and deglucuronidation and desulfation of the metabolites takes place before excretion [43,119].
The ratio of the different isoflavones is different in bile and urine. Daidzein is found in higher concentrations than genistein in urine, and a study of daidzein and genistein metabolites in blood and urine after ingestion of soy flour showed that the recovery in urine was higher for daidzein than for genistein, even though the composition ratio of genistein:daidzein was 1:7. This indicates that genistein and its conjugated metabolites are excreted into the bile rather than urine to a larger extent than daidzein, and undergo more enterohepatic circulation [43]. Genistein is more susceptible to degradation by intestinal microflora compared to daidzein due to its 5-OH group, and less genistein may therefore be available for absorption resulting in lower urinary recovery [14,149]. Unknown metabolites of genistein may be present in urine, and therefore an underestimation of the excreted amounts of genistein takes place [43,53]. Plasma genistein concentrations are consistently higher than daidzein concentrations, which can be explained by the excretion into the bile mentioned before, but also by the fact that daidzein is more extensively distributed in tissues in the body [111]. Furthermore, plasma daidzein has a faster clearance rate than genistein, and is therefore found in plasma for a shorter time [103,144].

4.1 Isoflavone metabolites

Generally there is a low recovery of isoflavones in urine, and the faecal excretion is also low, indicating that a large percentage of dietary isoflavones are metabolised beyond deglycosylation [112,134]. In a study by Watanabe et al. recoveries of daidzein and genistein in urine and faeces were 55% and 20% respectively after ingestion of baked soybean powder [144]. Because of large interindividual variations in gut microflora, great variation in the amount of metabolites are found in different individuals, and the diet has an influence on the degree of metabolism of isoflavones [134,151].

The formed metabolites differ in structure and estrogenic effect from the parent molecule. Equol (7-hydroxy-3-[4’-hydroxyphenyl]-chroman) and o-desmethylangolensin (ODMA) are the main metabolites of daidzein, and dihydroadaidzein and 4-hydroxyequol are also formed. ODMA is weakly estrogenic (appr. one third of daidzein affinity to ERα) and equol has a higher estrogenic effect than daidzein (twofold higher affinity to ERα). Fewer metabolites of genistein are known but one example is p-ethyl phenol, which is hormonally inert [38,108,134,151].

Equol is the most abundant and potent metabolite of daidzein and is structurally similar to estradiol [140]. The two enantiomeric forms, S- and R-equol, show different binding affinities to ERs [151]. Human intestinal bacteria show enantiomeric specificity in their production of equol, and only produce S-equol [117,151]. The ability to produce equol is affected by race due to differences in gut microbiota. Approximately 30% of the population in America and Europe are able to produce equol, whereas in Asia the proportion is 50% [89,91,111,140]. The conversion of daidzein into equol might be affected by the habitual diet. It has been shown in some studies that a high carbohydrate, fibre and polyunsaturated fat intake increases the rate of conversion, and there is a higher proportion of equol producers among vegetarians than non-vegetarians [89,104,111,118]. Other studies have correlated
high equol production with high fat and meat intake, and some have shown that fibres have adverse effect on isoflavone absorption. Yet other studies fail to show an effect of fibre and isoflavone intake on the ability to produce equol [60]. The contradictory results may be due to variations in age, diet and intestinal flora of the subjects [103]. Pre- and probiotics might also have an influence on production of equol because of their effect on gut microflora [130]. There is a time delay of 6-8 hours after ingestion before equol appears in plasma, which suggests that the metabolism takes place in the colon [89,111]. A study revealed a higher plasma concentration of equol after ingestion of glycosides compared to aglycones, as they are present in the colon and have a longer transit time [156]. A study of the effect of food matrix showed a higher urinary equol excretion when ingesting tempeh, which is a fermented solid food matrix, compared to soy milk and textured vegetable protein (TVP). A solid food matrix might protect daidzein from degradation until it reaches the large intestine, where it is metabolised to equol [28].

The isoflavones biochanin A and formononetin are demethylated, to a great extent, in vivo into daidzein and genistein respectively [44,72]. This either happens by microbial demethylation or by oxidative demethylation catalysed by cytochrome P450 enzymes [72]. Therefore, a proportion of the biochanin A and formononetin ingested is found as daidzein and genistein in plasma, and generally, the concentrations of biochanin A and formononetin are lower than daidzein and genistein [44,111].

5 Bioavailability

5.1 Aglycones vs. glycosides

Studies have shown that isoflavones from fermented soy products are more available to humans than unfermented products, which is thought to be due to the formation of aglycones from glycosides in the fermentation process [15,51]. In a study by Okabe et al. a higher maximal concentration (60%) and area under the plasma concentration-time curve (AUC) (20%) were observed for the aglycone-rich soybean powder compared with the glycoside rich form [89]. Cassidy et al. compared pharmacokinetic parameters of daidzein and genistein after ingestion of tempeh (rich in aglycones) and TVP (rich in glycosides) and found higher maximal concentrations (10-114 %) and AUCs (20-120 %) after tempeh intake [15]. A study on three different soymilks (untreated, fermented and enzymatically treated) showed that soy aglycones were absorbed faster and in higher amounts than their glucosides [51]. Izumi et al. showed higher concentrations of isoflavones in plasma after both single-bolus dosage and long term intake of aglycone- or glycoside-rich supplements based on soybean extract [47].

Xu et al. however couldn’t show differences in bioavailability when subjects were fed TVP, tempeh, tofu and cooked soybeans, even though the four foods contained different amounts of aglycones and glycosides [150]. Richelle et al. conducted a study where a prehydrolysis process was carried out, and the bioavailability of the isoflavones was studied. The absorption patterns were very similar for
the aglycone and glycoside soy drink ingested, and there was no delay observed in the absorption of glycosides [101]. These results indicate that although the deglycosylation step is essential for absorption, it might not be rate-limiting, even though this has been suggested in other studies [85,101]. These include studies by Setchell et al. and Izumi et al. who have shown that the time to reach maximum plasma concentration is longer for glycosides than for aglycones [47,111].

Even though a higher bioavailability of aglycones is expected, a few studies have shown the opposite including studies by Rufer et al. and Setchell et al. Both studies used pure isoflavones, which might show different behaviour compared with food matrices, as interactions between the mixtures of isoflavones are not found as in soy products. Furthermore, a small number of subjects were included in the studies decreasing their power [105,111].

The higher bioavailability of glycosides is unexpected as the aglycones can be directly absorbed without hydrolysis, and the hydrophilic properties and larger molecular weight of glycosides is thought to result in poorer absorption in the gut [47]. It is known that apolar compounds are more easily absorbed over the cellular membrane, and a study of isoflavone uptake in Caco-2 cells showed that daidzein and genistein aglycones were taken up more easily into the cells, and the glucosides were not transported through the cell monolayer [79].

The stability of aglycones and glycosides may also affect their degree of absorption. Differences in their chemical structure result in glycosides being more stable and water soluble, and are therefore to a large degree delivered to the β-glucosidases in the large intestine and absorbed. The aglycones are less stable against bacterial degradation and if allowed to reach the large intestine may, to a larger extent be degraded into known or unknown metabolites prior to absorption. The sugar moiety of the glycoside might protect against this form of degradation [85,105]. Inter-individual differences in intestinal conditions leads to variation in the degree of degradation of isoflavones and this contributes to the disparities seen in bioavailability.

Other factors that might contribute to the contradicting results are differences in study design, food matrix and composition of formulations. There is no consensus on linearity between dosage and bioavailability, and it is therefore difficult to compare studies using different doses [105]. Some studies are cross-over studies whereas others are not, and the inter-individual variations therefore affect results to different extents, and might play a more important role in bioavailability than the molecular form of isoflavones. Aglycones and glycosides bind differently to food components eg. proteins, and bioavailability is influenced by whether the isoflavones are administered in a food matrix or as pure compounds [98]. These differences make it difficult to determine the exact effect of the attached sugar moiety, and suggests that isoflavone metabolism is more complex than it appears.

Active transport of isoflavone glycosides has not been proven, and a study has denied the transport of flavonoids by the sodium dependent glucose transporter (SGLT1), which has been shown to transport quercetin glycosides in other studies [56]. It has been shown that attachment of a glucose
group enhances the bioavailability of quercetin compared to aglycones supporting the existence of a glucose transporter [42]. Even though this has not been shown for isoflavones similar mechanisms might exist for their absorption and future research might lead to the discovery of yet unknown transporters. If these are present, they would be expressed differently in individuals, and this could partly explain why there are differences between the studies. However, no isoflavone or quercetin glycosides are found in human plasma so hydrolysis must take place before transport to the liver [34,105].

It has been shown that aglycones are absorbed faster and in greater amounts than glycosides, which is due to their hydrophobic character and lower molecular weight. A hydrolytic step is not needed prior to their absorption and they are absorbed in the upper part of the gastro intestinal tract. The contradictions in the field suggest that it is a challenge to simply compare aglycones and glycosides as many other factors affect absorption and bioavailability including food matrix, isoflavone source, intestinal bacteria and background diet.

5.2 Effect of matrix

The composition of isoflavones in supplements or foods varies depending on the origin of the isoflavones and processing methods. The absorption of compounds is affected by the solubility of a substance in the intestine, and therefore it is expected that isoflavones from a liquid matrix are absorbed faster than isoflavones from a solid food matrix. This was shown in a study where faster absorption rates and earlier peak serum concentrations were seen for soy milk compared with TVP [15]. de Pascual-Teresa et al. studied the absorption of isoflavones after ingestion of cookies, chocolate bars or juice, and the maximum concentrations in serum were the same for all three matrices. The observed differences were a faster absorption of genistein following consumption of the juice compared to cookies and chocolate bars, as well as a lower total urinary recovery of genistein following ingestion of juice. The differences in the results between the groups didn’t reach statistical significance, but the data suggested that absorption of isoflavones may be affected by the food matrix [91]. In a study looking at urinary recovery of isoflavones after ingestion of different soy foods, no differences between soy milk, tempeh and TVP were seen in total urinary isoflavone excretion. However, when looking at the individual isoflavones and subject groups food effects could be found [28]. Franke et al. also studies urinary excretion of isoflavones after ingestion of four different soy foods, and absorption patterns were different for the studied isoflavones. In accordance with some previous studies, they found higher bioavailability for soymilk, but for the protein powder drink the bioavailability was lower than the solid foods. Differences were also found between the two solid foods, indicating that not only the consistency of the food matrix but also the composition (eg. fat and fibre content) influences bioavailability [29].

Many supplements containing isoflavones from various sources are available, but it is unknown whether they have the same clinical effect as isoflavones from phytoestrogen-rich foods. Furthermore, it is not known whether possible differences in effects are caused by differences in
bioavailability, metabolism or other factors [111]. Studies comparing bioavailability of isoflavones from supplements and food matrices are limited, and show different results. Gardner et al. showed that the bioavailability of isoflavones was higher for soy foods than isoflavone supplements. Even though the dosage of isoflavones was lower when provided via soy foods, a higher serum concentration of genistein and daidzein were seen when ingesting soy foods compared to tablets. The exact isoflavone content in the soy foods was not chemically determined but estimated from databases, and the isoflavone form was not known and could be very different from the 98% glucoside content in the supplements [30]. Vergne et al. compared bioavailability of daidzein and genistein after ingestion of a soy supplement and soy based cheese, and found a higher bioavailability for supplements. The total isoflavone intake was similar for the two formulations, but there were differences in aglycone content (highest in cheese) and the ratio between daidzein and genistein [139]. Differences in aglycone content and ratios between isoflavones could have a great influence on the results. Another factor which could influence bioavailability in these cases is the excretion of bile. This is induced when ingesting protein and lipids, and has been shown in an vitro study to potentially increase bioavailability [142]. As the isoflavones are found in a more pure form in supplements and are not bound to other components as in a food matrix, a higher bioavailability from supplements could be expected. However, synergistic effects between isoflavones and other components may be present in food matrices affecting bioavailability of isoflavones in yet unknown ways.

It is not possible to draw clear conclusions on the differences in bioavailability between supplements and isoflavone rich foods, and lacking standardisation of biological products is a further challenge as large variations in isoflavone content are seen in commercial preparations, and large discrepancies between declared and actual content have been found [111,139].

5.3 Intestinal microflora

Endogenous glucosidases in the small intestine have been shown to be responsible for hydrolysis of isoflavones in germ-free rats [14]. This suggests that hydrolysis of glycosides is to a certain extent carried out by mammalian glucosidases, but glucosidases produced by gut microflora are also an important source and the gut microflora thereby has an influence on the bioavailability of isoflavones [104,134]. Enterococci display high β-glucosidase activity followed by Lactobacillus, Bacteroides, and Bifidobacterium. These microorganisms are present in high numbers in the small and large intestine [134]. Gut microflora are also responsible for metabolism and degradation of isoflavones and the extent of this is reflected in isoflavone recovery in faeces. Xu et al. showed that urinary recovery of isoflavones was twice as high in high faecal excretors compared to low excretors [149]. The bacteria in the high excretors are not as effective as in the low excretors and the isoflavones are therefore more available for absorption, and the intestinal microflora thereby has an impact on isoflavone bioavailability. There are large inter-individual differences in the absorption and excretion of isoflavones, which might be due to the influence of differences in gut microflora populations as well as past exposure to isoflavones and glucosidase production [53,101,156].
Probiotics are able to increase populations of bacteria that are beneficial to host health, and have been shown to alter enzyme activity and contribute to conversion of glycosidic isoflavones into aglycones in soy products [84]. It is expected that the bioavailability of isoflavones would be increased by ingestion of probiotics as it increases bacterial populations in the gastrointestinal tract and enzyme activity [61]. In a study by Cohen et al. there was a 40% reduction in urinary excretion of isoflavones after probiotic administration indicating increased circulating levels of isoflavones [21]. Nettleton et al. and Larkin et al. however failed to show increased plasma levels of phytoestrogens after administration of probiotics, even though the gut microflora was significantly altered [61,84]. It cannot be excluded that different bacterial strains, higher levels of probiotic bacteria or longer-term intake of probiotics might show different results. Amount of isoflavone intake may also affect results, as hydrolysis may not be a rate-limiting step at low doses.

Gut transit time (GTT) may also have an effect on the bioavailability of isoflavones, as it affects the degree of metabolism. The longer the isoflavones are in the gut, the more time there is for microorganisms to degrade them and a smaller amount is available for absorption [38]. Zheng et al. also showed a higher absorption of isoflavones in women with a rapid GTT and a low isoflavone degradation phenotype [155].

5.4 Effect of diet

Fibres might have an influence on the absorption of isoflavones, and a study by Piazza showed an increase in isoflavone absorption upon ingestion of inulin. This is thought to be due to a stimulation of bacterial growth and thereby increased hydrolysis of glycosides into aglycones [93,140]. Tew et al. however showed that the total urinary excretion of genistein was reduced with increased fibre content in the diet. They suggest that the insoluble fibres bind to the isoflavones and prevent their absorption throughout the gut. Furthermore, the insoluble fibres bind water and thereby cause bulking of the luminal content of the intestine, and a dilution of the gut microflora responsible for the hydrolysis of the isoflavones prior to absorption [131]. Other dietary compounds such as protein and fat may also affect the absorption of isoflavones. A short-term study failed to show differences in bioavailability even though the protein and fat content varied in the diets. This may be because the study was too short, hence a longer term study would be better suited to show whether diet has an effect on bioavailability through effects on gut microflora [150].

Several studies have shown that a daily intake of isoflavones results in reduced bioavailability, and the explanation for this is not known. It may be due to the substrate concentration exceeding the hydrolytic capacity, rate-limiting uptake of aglycones or the isoflavones inducing their own metabolism [8,69,115]. Others however fail to show an effect of habitual intake. Wiseman et al. demonstrated that a habitual intake of soy isoflavones did not significantly affect concentrations of isoflavones and their metabolites in plasma, urine and faeces. Apart from an increase in β-glucosidase activity in the subjects consuming high soy diets, there doesn’t seem to be an effect of habitual soy intake on bioavailability [145]. In a study by Lampe et al. there was no difference in isoflavone
excretion between short- (3 days) and long-term (1 month) intake groups of premenopausal women [60].

5.5 Conclusion on bioavailability

Bioavailability has been shown to depend on many factors. The form isoflavones are in upon ingestion affect bioavailability where most studies have shown that the aglycones are more bioavailable than the glycosides as the hydrolysis step in the intestines is not needed [47,89]. No clear conclusions can be drawn regarding the effect of food matrix on bioavailability, but there is a tendency that it is higher for liquids compared to solid foods [16].

Gut microflora has a big impact on bioavailability and large inter-individual differences are seen. Even though clear results have not been found, the composition of habitual diets (fibre, fat, protein) is suggested to affect bioavailability through influences on gut microflora, and the extent of daily intake of isoflavones might also have an impact.

Many clinical trials have been carried out studying bioavailability of isoflavones, but large differences in the study designs and participant number make it difficult to draw clear conclusions. Differences in gut microflora, GTT and degradation phenotype may be a large contributor to differences in absorption and blunt the effects of other studied factors (matrix, aglycone/glycoside etc.).

6 Safety

The use of isoflavones as an alternative to HRT is becoming increasingly popular. When using isoflavone supplements there is potential of ingesting large amounts of isoflavones per day; much larger than doses obtained by isoflavone containing foods. This makes it important to assess the safety of ingesting large amounts of isoflavones daily.

In a 2-year study including healthy menopausal women, Steinberg et al. evaluated the safety of supplements derived from soy hypocotyl containing mainly daidzein and glycine in doses of 80 or 120 mg/day. They evaluated clinical blood chemistry measures (including complete blood count, thyroid and liver function, estradiol and lipid profiles), adverse events and did a physical examination (including blood pressure, endometrial thickness and mammogram). No statistical differences were found in blood chemistry markers except for urea after 2 years intervention, which remained within the reference range, and no differences were observed in the physical examinations. Two adverse events were reported, but the incidences were not different from those predicted from statistical probabilities, and their overall conclusion was that 2 year intake of isoflavones from soy hypocotyl is safe [125]. A study by Nahas also found use of soy isoflavones for 10 months in postmenopausal women safe as no serious adverse events were reported and clinical outcomes including endometrial thickness, mammography and hormonal profiles did not change as a result of soy extract supplement.
intake [81]. Powles et al. carried out a safety study of isoflavones from RC and found that they were safe and well tolerated by women with a family history of breast cancer. After 40 mg daily intake during 3 years no significant differences in breast density and endometrial thickness were seen between the isoflavone and placebo group [96]. Several more studies of varying size (19-138 subjects), isoflavone dose (36-92 mg/day) and duration (6-36 months) have also evaluated isoflavone intake as safe based on mammographic breast density, endometrial thickness and reports of adverse events [7,71,92,106].

There are also studies that report of serious adverse effects after long-term intake of isoflavones. Unfer et al. conducted a large 5-year study in healthy postmenopausal women where they observed endometrial hyperplasia in 3.8 % of the subjects in the intervention group compared to 0 % in the placebo group. No cases of endometrial hyperplasia were observed in the intervention group halfway through the study (after 30 months) and no cases of endometrial carcinoma were observed during the study period. It should be noted that the daily isoflavone dose was 150 mg which is about triple the daily intake of Asian populations [136]. In a smaller study by Wolff et al. 32 postmenopausal were given 80 mg/day of RC isoflavones during six months, and at the end of the study endometrial alterations were seen in three women. It should be noted that this study has a small study population and there was no placebo group for comparison [146].

It is difficult to determine the most appropriate isoflavone dose and study duration for safety studies. In some of the previously mentioned studies the dose of isoflavones is higher than recommended values, which might induce cellular changes that would not have occurred at lower doses [136]. Soy foods and isoflavone preparations have different compositions of isoflavones, and the results from safety evaluations in one study cannot necessarily be used to establish safety profiles of other isoflavone preparations. Despite differences in study designs, most studies suggest minimal health risks with isoflavones supplementation and epidemiological data support the claim that isoflavone intake is safe as a lower rate of endometrial cancer is seen in Asian women compared to Western societies [10,125]. Isoflavones modify ERs in different ways depending on the structure of the receptor, which means that they can exert agonistic, partial agonistic and antagonistic effects. Because individuals have different distributions of ERs, they can also have different sensibilities towards the isoflavones, and this can affect the results of safety evaluations [136].

7 Physiological effects of isoflavones

7.1 Menopause

Isoflavones have estrogenic effect because they have structural similarities to estrogen and similar molecular weights. Estrogenic effects are reached when a steady-state plasma concentration of isoflavones of 50-800 ng/ml is reached, which corresponds to concentrations found in Asian populations [9]. The 4’ hydroxyl group in the isoflavone structure is the binding site for ERs, and
therefore the structures of the different isoflavones have an impact on their estrogenic effect [111]. Morito et al. showed that methylation of the hydroxyl group reduced the affinity of the isoflavones towards ER, as formononetin and biochanin A bound less strongly to ER $\alpha$ and $\beta$ compared to genistein [75]. There is also a difference in the binding affinities of genistein and daidzein. Genistein has a much higher binding affinity to ER$\beta$ than daidzein, which is due to a decrease in polarity of the ester functionality caused by interactions between the proximal hydroxyl group and the carbonyl group [80].

The effect of isoflavones on ERs are similar to SERMs, and they show agonistic or antagonistic effect depending on the concentration of endogenous estrogen and the type of ER [115,151]. In case of a high level of endogenous estrogen, phytoestrogens bind to ERs and reduce the effect of endogenous estrogen, and thereby induce antagonistic effects. On the contrary, if there are low levels of endogenous estrogen in the body, phytoestrogens act as stand-ins for estrogen and exert agonistic effects [66,80].

It has been shown in several studies that equol producers have greater benefits of isoflavone containing diets compared to non-equol producers [113,118]. This is assumed to be because S-equol is more estrogenic compared to daidzein and other metabolites of daidzein, as it has similar binding affinities to ER$\alpha$ and ER$\beta$ as genistein, which is the most potent soy isoflavone [130,151]. The binding affinity of S-equol to ER$\alpha$ is relatively poor, which is also the case for the ability of R-equol to bind to both types of ERs [117]. It is unique for S-equol that it possesses estrogenic effects as well as antiandrogen action, which might be the reason why a greater effect of isoflavone diets is seen in equol producers [118]. Equol binds ER$\beta$ receptors with 20% the affinity of 17$\beta$-estradiol, but is excreted in amounts 10 to 1000 times greater than endogenous estrogen depending on the diet [46,117]. A larger proportion of equol is found in serum in its free form compared to daidzein and estradiol [151]. Only 50% of equol is protein bound, compared to 95% of endogenous estrogen. Only the unbound compounds can bind to ERs, and this difference means that the biological potency of equol is enhanced compared to estrogen [117]. Furthermore, there are differences in the renal clearance of the different isoflavones and metabolites, and equol remains in plasma for a longer time than genistein and daidzein which further enhances its biological effect [118,156].

The estrogenic effect of isoflavones is thought to help relieve menopausal symptoms including hot flushes. In a study by Lipovac et al. administration of 80 mg/d of RC isoflavone aglycones resulted in a 74% reduction in menopausal symptoms (hot flush/night sweat frequency and Kupperman index) in postmenopausal Austrian women [67]. A 78% reduction in menopausal symptoms was seen in postmenopausal Ecuadorian women who received RC supplements for 90 days. A placebo effect was seen in this study, as a 23% reduction in Kupperman index was seen after administration of placebo [40]. Many of the studies of menopausal symptoms have outcome measures based on subjective evaluations, including the two mentioned studies. In one study sweat secretion over 24 h was measured in menopausal women suffering from menopausal symptoms. A mean reduction of 15% in hot flush frequency and 32% in intensity of hot flushes was found after daily intake of RC during 12 weeks [59].
There are also many examples of studies that either fail to show an effect or show similar effects in intervention and placebo groups. In a study by del Giorno et al. similar reductions in menopausal symptoms were seen in groups receiving 40 mg isoflavones and placebo [32]. Tice et al. investigated the effect of two RC supplements providing 82 mg and 57 mg isoflavones on the frequency of hot flushes in postmenopausal women. They found similar reductions of around 37% in the intervention and placebo groups [133]. These results might be a reflection of the placebo effect where expectations influence perception, which becomes very apparent when subjective outcome measures are used.

Isoflavones are thought to help relieve menopausal symptoms through their estrogenic effects, and many studies have investigated the effects of phytoestrogens on menopausal symptoms with varying results. There are many factors contributing to the contradicting results in these studies, and heterogeneity between study designs (isoﬂavone preparations, outcome measures etc.) is a major one. The lack of clear results might also partly be explained by the differences in ability to produce equol. Equol producers might have the most gain of isoflavones in relieving hot flushes, and there has been made no distinction between producers and non-producers in most studies [114].

7.2 Osteoporosis

When women reach menopause the lack of estrogen production results in an imbalance in bone metabolism where the rate of bone resorption exceeds the rate of bone formation. This leads to a decrease in bone mineral density (BMD) and an increased risk of fractures and development of osteoporosis. Phytoestrogens might be able to improve the imbalance due to their estrogenic effects. In vitro studies and studies on rodents have shown that isoflavones reduce bone resorption and therefore potentially could have antiosteoporotic effect [114]. ERs have been found in osteoblast and osteoclast cells, and it has been shown that the action of daidzein is mediated through ERs in these cells, resulting in stimulation of osteoblasts and inhibition of osteoclasts [31]. Occhiuto treated ovariectomized rats with RC isoflavones and showed a reduction in the number of osteoclasts. The treated rats had increased bone mineral content and femoral density as a result of reduced bone resorption [88].

The antiosteoporotic effect has also been investigated in several human studies, but contradicting results exist. Epidemiological and cross-sectional studies have shown a reduction in fractures and increases in BMD with increasing intake of soy products [73,152]. Some intervention studies have shown an improvement in BMD after administration of phytoestrogens, while others have failed to show a beneficial effect of isoflavones on BMD. Atkinson et al. investigated the effect of 1 year daily supplementation of RC isoflavones (appr. 40 mg/d) on bone density in women. Isoflavone supplementation resulted in a smaller decrease in spine bone mineral content (BMC) and BMD compared with placebo; percentage changes in BMD were - 1.08 % and -1.86 % for treatment and placebo groups respectively. A similar pattern was seen for the hip but differences were not significant, probably due to the hip consisting primarily of cortical bone which has a slower bone
Examples of studies that fail to show a positive effect of isoflavones on bone include a study by Levis et al. where postmenopausal women received 200 mg/d isoflavones from soy during 2 years. No significant differences were found in spinal (-2.0% vs. -2.3%), femoral neck (-2.2% vs. -2.1%) and total hip (-1.4% vs. -1.2%) BMD between the isoflavone and placebo group [64]. Tai et al. also failed to show differences in rates of bone loss between 2 year daily intake of soy isoflavones (300 mg/d) or placebo, evaluated from lumbar spine and femoral neck BMD values as well as bone turnover markers [126]. The reason for conflicting results in the intervention studies might be differences in study designs including dosage, product form and study duration. Especially the source of isoflavones may have a significant influence on the antiosteoporotic effects as intervention studies using RC generally show more positive effects than soy, which may be due to the unique composition and higher content of isoflavones in RC. Furthermore, it might be important in such studies to make a discrepancy between equol producers and non-producers as the former may have a more pronounced effect of isoflavone treatment on bone state [31,114].

Formononetin, which is found in high concentrations in RC, has been found to have an increased potency on osteoblast function compared to daidzein, and exerts its action by activating the p38 mitogen-activated protein kinase dependent pathway. Gautam et al. have shown that formononetin does not exert its action through ERs in the same way as daidzein, and is purely osteogenic [31]. Tyagi et al. showed that formononetin restored bone in osteopenic rats by enhancing bone formation [135]. Due to a high degree of demethylation in the gut, the concentration of formononetin in plasma is however lower than daidzein and genistein, and the effect on bones is mostly from the two latter isoflavones and the metabolite equol [111].

Through their estrogenic effect, isoflavones are thought to have a beneficial impact on bone health in postmenopausal women with an imbalance in bone metabolism. Isoflavones have been shown to inhibit osteoclasts and stimulate osteoblasts in in vitro and animal studies. Soy isoflavones are the most extensively studied and results are inconsistent, but the few studies on RC show very promising results, which is most likely due to its high content of formononetin [6,17,52,59].

7.3 Cancer

Four Asian retrospective epidemiological studies have shown that when ingesting isoflavones early in life they exert a protective effect towards breast cancer. Korde et al. investigated correlations between soy intake and risk of breast cancer in Asian American women, and found an inverse relationship which was strongest for childhood soy intake (RR: 0.40), but was also seen for adolescent and adult soy intake. Lee et al. found a protective effect of soy protein and isoflavone intake against premenopausal breast cancer in Chinese women. A high soy food intake during adolescence was associated with a 43% reduced risk of premenopausal breast cancer [55,63,121,147]. Thanos et al. found similar results among non-Asians (Canadian study). They found a 20% reduction in breast cancer risk for a high isoflavone intake during adolescence [132]. These findings are further
supported by animal studies. Lamartiniere et al. found a reduction in chemically induced mammary cancer in rats after prepubertal exposure to genistein [58].

Studies have shown that daidzein and especially genistein have anticarcinogenic effect because they inhibit the proliferation of cancer cells. The exact mechanism behind is unknown but is thought not to be exclusively hormonal and include several properties like activation of ERs, inhibition of growth promoting steroid hormones, antioxidative and antiangiogenic properties [47,112,140]. Pino et al. have shown that isoflavone intake is associated with an increase in sex hormone-binding globulin (SHBG) in postmenopausal women, especially in those women having a low level of SHBG at baseline [94]. An increase in SHBG decreases the free fraction of estradiol in plasma and thereby its biological activity, and this can partly explain the anticarcinogenic properties of isoflavones [1].

The anticarcinogenic effects of isoflavones are different in equol-producers and non equol-producers, as there is an enhanced expression of estrogen-responsive genes in equol-producers [151]. Furthermore, equol-producers might have a more favourable hormonal profile. A study by Duncan et al. showed that equol-producers had lower plasma levels of sex hormones and higher levels of SHBG, which is a hormonal pattern, associated with a lower cancer risk [26]. The bacteria involved in converting daidzein to equol, also reduces the enterohepatic recycling of reproductive hormones [151].

However, concern has been raised about isoflavones being harmful to breast cancer patients. Several studies have evaluated the effect of isoflavones on breast-cell proliferation, and an increase in cell proliferation was not found in response to isoflavone intake by Hargreaves et al. and Cheng et al. [18,37]. Even though these are small and short-term studies, the results are positive as opposed to the increased cell proliferation seen with HRT [25,78]. Epidemiological studies of this issue have also been done, and an analysis of three cohort studies carried out by Nechuta et al. showed that isoflavone intake by cancer survivors was associated with at reduced risk of breast cancer recurrence and mortality [82].

Epidemiological studies suggest that ingestion of isoflavones early in life has a protective effect against cancer later in life, and there are animal studies that support these findings. The exact mechanisms behind potential beneficial effects are hard to determine because of many pathways being involved in cancerous diseases and isoflavones potentially acting in many different ways (hormonal, antioxidative etc.) It is difficult to make definite conclusions on whether phytoestrogens have an anti-carcinogenic effect. Even though there is much evidence to support this hypothesis, the nature of the disease makes it difficult to investigate the effects, as there is potential for many biases. Hence, a greater number of larger scale and longer-term studies are needed.
7.4 Diabetes

The prevalence of diabetes and metabolic syndrome is rapidly increasing worldwide. Isoflavones could potentially have a beneficial effect on these conditions by affecting lipid metabolism and glucose homeostasis.

Several animal studies have shown promising effects of isoflavones on plasma lipid profiles and glucose levels. In a study with Zucker diabetic fatty rats lower plasma concentrations of total cholesterol, triglycerides (TG) and free fatty acids (FFA) were seen after administration of isoflavone rich soy protein for ten weeks. Furthermore, lower levels of fasting blood glucose were detected in the soy-fed group throughout the treatment period [27]. Nordenstoft et al. carried out a study with KKAy mice and found a 38% reduction in TG and 31% reduction in total cholesterol after 9 weeks feeding with isoflavone rich soy protein. The soy protein was also shown to have antihyperglycemic and antihyperinsulinemic properties as a 64% reduction in plasma glucose and a 85% reduction in insulin was seen [87]. This shows that the animals have become more sensitive towards the action of insulin, which is positive in the treatment of diabetes.

Many trials have been conducted to investigate the effect of soy proteins on plasma lipid profiles in humans [5,99,129]. A meta-analysis of 38 studies has shown that soy proteins reduce serum concentrations of total cholesterol (9%), LDL cholesterol (13%) and TG (11%) in humans. The effect is most pronounced in hypercholesterolemic subjects [5]. Another meta-analysis of 11 studies compared cholesterol lowering effects of isoflavone enriched and depleted soy protein. Enriched soy protein significantly reduced serum total (1.77%) and LDL (3.58%) cholesterol compared to depleted soy protein when ingesting 100 mg isoflavones per day [129]. These studies have created the foundation for the health claim from The Food and Drug Administration (FDA), that 25 grams of soy protein a day may reduce the risk of heart disease. In an intervention study by Hermansen et al. reductions in LDL cholesterol, LDL/HDL cholesterol ratio and TG levels of 10%, 12% and 22% respectively were found in type 2 diabetic subjects supplemented with isoflavone-rich soy protein for 6 weeks. No effect of soy protein was seen on glucose and insulin responses [39]. Studies using isoflavone sources other than soy protein have also shown lipid lowering effects. A 2 year study including postmenopausal women who were administered 50 mg/d isoflavones as RC supplements showed a 12% reduction in serum LDL cholesterol [20]. Clerici et al. conducted a study with soy germ-enriched pasta which is rich in isoflavone aglycones. Intake of this pasta was compared with conventional pasta in hypercholesterolemic adults and showed reductions in total and LDL cholesterol of 7.3% and 8.6% respectively, and the effect was more pronounced in equol producers [19]. It is known that estrogen has an impact on lipid profiles, and the positive effects of isoflavones might be due to their estrogenic properties.

There are however also studies that fail to show an effect of isoflavones on the lipid profile. Administration of a RC supplement to postmenopausal women with moderately elevated plasma cholesterol levels didn’t significantly alter plasma levels of total, LDL and HDL cholesterol as well as TGs [45]. Lichtenstein et al. showed that isoflavones added to soy and animal protein diets did not
have an effect on plasma lipid levels in moderately hypercholesterolemic subject [65]. As the subjects in these studies only had moderately elevated levels, the possible effects of the intervention may be difficult to detect.

Glucose metabolism impairment is an important feature of the metabolic syndrome and the effects of isoflavones on glucose metabolism is another way in which isoflavones might affect the risk of developing type 2 diabetes. Zhang et al. found in a meta-analysis that soy isoflavone supplementation reduced body weight, blood glucose and insulin in postmenopausal non-Asian women. The effect on body weight and insulin levels was most pronounced in women with normal body weight (BMI < 30) and the best effect on blood glucose is obtained with long-term and lower dosage of isoflavones [154]. In a one year study by Squadrito et al. the effect of pure genistein was investigated in postmenopausal women with metabolic syndrome. They found a mean difference change in HOMA-IR of -1.4 and a significant reduction in blood glucose and insulin levels after 12 months intervention. Beneficial effects on lipid profiles and blood pressure were also found in this study. The study showed that genistein treatment significantly improved features of the metabolic syndrome, and after the intervention only 30% of the women in the genistein group met the criteria of metabolic syndrome [124].

However, contradictions exist regarding the effect of phytoestrogens on glucose metabolism. A meta-analysis of 10 intervention studies showed that daily ingestion of isoflavone mixtures did not reduce serum levels of glucose in non-Asian women [100]. Gopert et al. failed to find an effect of soy protein isolate on markers of glycaemic control after 8 weeks intervention in type 2 diabetics who did not manage their disease with medication. Fasting and postprandial glucose and insulin levels, HbA1C and HOMA-IR were not reduced after the intervention compared to intake of milk protein [33]. The contradictory results regarding glucose homeostasis may be caused by differences in baseline values before intervention and study duration, as shorter studies generally fail to show effect.

Other dietary compounds having a more pronounced effect on lipid metabolism than isoflavones might contribute to the contradictory results. It is not clear whether the positive effects on lipid profiles and glucose homeostasis seen in studies with soy protein are due to isoflavones or other components of soy (proteins, fibers, saponins etc.) or a combination. In a meta-analysis it was shown that depleted soy protein significantly lowered LDL and total cholesterol and increased HDL cholesterol compared with animal protein, which suggests a beneficial effect of the proteins in soy [129]. There is however also evidence that fibre and isoflavones in the soy have lipid lowering properties [39]. All this suggests that several compounds in soy have beneficial effect on plasma lipid profiles and there might even be synergistic effects [95,128]. Other factors potentially contributing to contradictions are differences in the ability of the subjects to produce equol, differences in plasma cholesterol states of subjects before intervention and the limited sample sizes in some of the studies, which might affect the ability to detect significant differences.

The exact mechanisms behind the effects of isoflavones on lipid and glucose metabolism are not clear and remain controversial. One way of action is thought to be through activation of peroxisome
proliferator activated receptors (PPARs). PPARγ is involved in numerous processes in the body including lipid metabolism, glucose homeostasis, insulin sensitivity and inflammation. Isoflavones may act as selective PPARγ modulators with an agonistic effect similar to endogenous ligands [143]. Mueller et al. showed in an in vitro study that isoflavones from RC, and especially their metabolites, are potent PPARγ binders and activators. Their maximal PPARγ activities range from 15-32% of the maximal activity of rosiglitazone, which is a synthetic PPARγ activator used for treatment of type 2 diabetes but with negative effects on lipid profiles and weight [76].

Isoflavones have also been shown to act directly on pancreatic β-cells. In vitro studies carried out by Liu et al. showed that genistein increased glucose stimulated insulin secretion by increasing adenylate cyclase activity and thereby elevating intracellular cAMP levels. This leads to activation of the cAMP/PKA cascade and increased insulin secretion [68]. Genistein is a potent inhibitor of tyrosine-specific protein kinases, and this effect may partially explain the effect genistein has on insulin secretion as tyrosine kinases have been shown to be involved in the regulation of islet β-cell function [4,123].

Many other mechanisms have been suggested including inhibition of α-glucosidase and glucose-6-phosphatase and reduction of intestinal glucose uptake [2,62,138].

Evidence suggests that soy protein has beneficial effects on the conditions associated with the metabolic syndrome including decreases in LDL and total cholesterol as well as blood glucose. These effects probably stem from several compounds in soy, and isoflavones might be a contributor, as effects have also been shown with RC supplements and pasta rich in isoflavone aglycones. A clear conclusion can however not be drawn as there are also studies failing to show beneficial effects of isoflavones on plasma lipid profiles and glucose levels, and this is most likely due to large between-study heterogeneity and the beneficial effects on the metabolic syndrome stemming from many compounds in the diet.

### 7.5 Cardiovascular disease

The metabolic syndrome is associated with an increased risk of coronary heart disease and cardiovascular mortality, and increasing age and menopause is associated with an increased risk of suffering from CVD [20,39]. Estrogen deficiency leads to unfavourable changes in lipid metabolism and an increase in serum cholesterol levels, which are risk factors for developing CVD. Apart from evidence for the beneficial effects of isoflavones on plasma lipid profiles described in section 7.4, there are other ways in which isoflavones affect the risk of CVD.

Endothelial NO has a vasodilator function and is involved in the regulation of blood flow and vascular tone. NO has also been shown to have anti-inflammatory and antiatherogenic effects, and decreased NO synthesis is partly responsible for the initiation of atherosclerosis [122,151]. Endothelial nitric oxide synthase (eNOS) is activated by estrogen, and it is of interest whether phytoestrogens can
activate eNOS in postmenopausal women with reduced estrogen production. Administration of RC isoflavones to human endothelial cells showed an increase in NO release and activation of eNOS. This is a genomic effect where an upregulation of eNOS expression is seen, similar to that induced by estrogen. The effect was enhanced when the isoflavones were provided together with menopausal concentrations of estradiol [122]. Joy et al. showed that equol is a potent activator of NO production in human endothelial cells, and documented that equol induces relaxation of aortic rings [49]. Inducible nitric oxide synthase (iNOS) is another form of NOS predominantly found in macrophages, and overproduction of NO by iNOS is associated with development of atherosclerosis. Studies have shown that daidzein, genistein and equol inhibit NO production and expression of iNOS, which may be part of the explanation for their protective effects [50,120].

Atherosclerosis is an inflammatory process that is associated with increased expression of certain genes including cytokines, growth factors and acute-phase proteins [9]. Isoflavones have been shown to inhibit the activation of transcription factor NF-κB that plays an important role in the expression of various genes related to inflammation [77]. C-reactive protein (CRP) is a marker of inflammation that is often elevated in patients with atherosclerosis. Serum concentrations of CRP were reduced by 42% in a study with soy germ pasta rich in isoflavone aglycones [19]. Isoflavones inhibit cell adhesion, alter growth factor activity and inhibit cell proliferation which are all factors involved in atherosclerotic lesions and plaque formation. These effects are believed to be mediated through inhibition of tyrosine kinases [4,97]. Oxidative modifications of LDL is also an important mechanism in atherosclerosis, and the antioxidant properties of isoflavones may reduce lipid oxidation, by acting as free radical scavengers through hydrogen/electron donation [151]. The number and position of the hydroxyl groups have an impact on the antioxidative effect of isoflavones, and some metabolites are more antioxidative than the parent molecules, eg. equol is a more effective antioxidant than daidzein and genistein [151]. Being an equol producer might therefore lead to a greater reduction of the risk of developing CVD.

Activation of PPARs is associated with an improved lipid profile as it increases HDL cholesterol, decreases circulating TG levels and shifts LDL cholesterol particle size to a less atherogenic form. PPAR activation may also exert antiatherosclerotic actions through other mechanisms including reducing oxidative stress, accumulation of advanced glycation end products and inflammation [48]. Phytoestrogens have been shown to be PPAR agonists, and may exert their antiatherosclerotic effect through this activation.

Apart from potential effects on plasma lipid profiles, isoflavones have also been shown to increase production of NO by eNOS (which has a vasodilator function), as well as inhibit NO production by iNOS (which is associated with development of atherosclerosis). Isoflavones are shown to reduce the expression of genes associated with inflammatory processes in atherosclerosis and prevent cell adhesion and proliferation through inhibition of tyrosine kinases. Their antioxidative effects limit lipid oxidation. All these factors contribute to the capability of isoflavones to lower the risk of CVD.
8 Conclusion

Research on the physiological effects of isoflavones has provided evidence of potential ameliorating health effects in several diseases including menopausal symptoms, osteoporosis, cancer and type 2 diabetes. They exert their beneficial effects through estrogenic activities and antioxidant properties as well as activation of PPAR pathways. Soy and RC are rich sources of isoflavones. The fact that HRT increases the risk of breast cancer has further augmented the popularity of isoflavones in use as a safer alternative treatment.

Isoflavones are largely metabolised in humans, and some of the metabolites have higher biological activity compared to parent molecules; for example equol, a metabolite of daidzein. Bioavailability is important to consider when studying the physiological effects of isoflavones. Studies have shown that the degree of absorption depends on many factors including food matrix, isoflavone form and background diet.

There are generally many contradictory results in studies of bioavailability and effects of isoflavones. The reason for this may be, in part, the source and treatment of isoflavones preparations. The presence of synergistic effects between isoflavones and other components in food has not been elucidated and synergistic components could be removed during extraction processes, moreover isoflavones may also become inactivated during processing [6]. Another challenge is the poor standardisation and uncheck claims of contents of biological products, which means that there are great variations between commercial preparations. Furthermore, large inter-individual differences are observed as a consequence of variations in intestinal microflora or genetics, leading to differences in absorption and metabolism of isoflavones. A study of polymorphisms in genes has shown that there is an association between polymorphisms in certain genes and conjugation patterns of isoflavones. These polymorphisms affect the activity of enzymes and transporters and therefore influence metabolism and bioavailability of isoflavones, and through this their biological activity [141].

There are ways in which bioavailability could be enhanced and potentially increase the effects of isoflavones. Addition of probiotics might have a beneficial effect through influences on gut microflora even though research has not shown a direct correlation between ingestion of probiotics and increased bioavailability. Microencapsulation is a technique used to stabilise food additives and pharmaceutical products, which might represent another useful method to increase the effects of isoflavones by enhancing residence time and obtaining more constant plasma concentrations [116].

Even though many studies have been carried out on bioavailability and physiological effects of isoflavones, it is still important to execute large, well-constructed human studies of longer duration, with focus on the specific composition of the isoflavone products. Most studies have used soy isoflavones and it is of interest to study the effects of RC as it is one of the richest sources of isoflavones with a unique isoflavone composition, and has shown promising effects on relieving menopausal symptoms and decreasing bone demineralisation [59]. It might also be valuable to take
account of genotype when investigating the bioavailability and potential beneficial effects of isoflavones.

Conflict of interests

The authors declare no conflicts of interests.

References


49. Joy, S., Siow, R.C.M., Rowlands, D.J., et al. The isoflavone equol mediates rapid vascular relaxation: Ca 2+-independent activation of endothelial nitric-oxide synthase/Hsp90 involving ERK1/2 and Akt


155. Zheng, Y., Lee, S.-O., Verbruggen, M. a, Murphy, P. a, and Hendrich, S. The apparent absorptions of isoflavone glucosides and aglucons are similar in women and are increased by rapid gut transit time and low fecal isoflavone degradation. *The Journal of nutrition* 134, 10 (2004), 2534–2539.
Bioavailability of isoflavones from fermented and unfermented red clover formulations

Authors: Dorthe Møller Sørensen
Address: Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Tage-Hansens Gade 2, DK-8000 Aarhus C, Denmark

Abstract

Background
Red clover is a rich source of isoflavones, which have been shown to have beneficial physiological effects. Bioavailability is a necessity for isoflavones to exert their biological activity and the purpose of this study was to determine the bioavailability of isoflavones from fermented and unfermented red clover formulations.

Method
Fifteen healthy women aged 19-39 years participated in a crossover trial and were each given five red clover formulations (fermented liquid extract, fermented freeze-dried extract in capsules, tablets and yoghurt and unfermented capsules) separated by a wash-out period of minimum 5 days. The red clover formulations were ingested immediately prior to a standardised breakfast meal, and blood samples were collected at baseline and at 2, 4, 6, 8, 12, 24 and 48 hours after administration. These were analysed for isoflavone content, thereafter the bioavailability was evaluated from calculated incremental area under the concentration-time curves (iAUC).

Results
Daidzein represented the highest concentrations in plasma followed by genistein and formononetin. Peak plasma concentrations of total isoflavones were seen after 6 hours for fermented liquid extract and unfermented capsule (0.24 and 0.23 µmol/L respectively). The fermented capsule showed a tendency for higher area under the curve for total isoflavones than unfermented capsule (iAUC0-24h: 1.21 ± 0.28 vs. 0.99 ± 0.12 µmol·24h/L, P = 0.36). For daidzein the bioavailability was significantly higher in the fermented liquid extract compared to the unfermented capsule (iAUC0-24h: 0.62 ± 0.07 vs. 0.27 ± 0.05 µmol·24h/L, P < 0.05). There was a tendency for the same picture when looking at formononetin, but differences were not statistically significant. For genistein the bioavailability was surprisingly significantly higher in unfermented capsule compared to the fermented liquid extract (iAUC0-24h: 0.61 ± 0.07 vs. 0.33 ± 0.05 µmol·24h/L, P < 0.05) and the fermented capsule (iAUC0-24h: 0.61 ± 0.07 vs. 0.35 ± 0.07 µmol·24h/L, P < 0.05). This might be due to differences in isoflavone composition in the formulations. There were no differences in area under the curve between the fermented liquid extract and capsule when looking at the individual and total isoflavone curves.
**Conclusion**

There is a tendency for a higher bioavailability of total isoflavones in the fermented red clover formulations compared to unfermented. No differences in bioavailability were observed between liquid and solid formulation of fermented red clover. Results are based on data from a small percentage of the total number of collected plasma samples, due to challenges with analytical equipment. A comparison of all five included formulations based on an adequate number of time points will be possible when all samples have been analysed.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC</td>
<td>red clover</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>AUC</td>
<td>area under concentration-time curve</td>
</tr>
<tr>
<td>iAUC</td>
<td>incremental area under concentration-time curve</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>TVP</td>
<td>textured vegetable protein</td>
</tr>
<tr>
<td>lex</td>
<td>liquid extract</td>
</tr>
<tr>
<td>fcap</td>
<td>fermented capsule</td>
</tr>
<tr>
<td>ufcap</td>
<td>unfermented capsule</td>
</tr>
</tbody>
</table>

**1 Introduction**

Isoflavones are phytoestrogen components present in the human diet, of which soy and red clover (RC) (*Trifolium pratense* L.) are abundant sources. RC is native to Europe and Western Asia, and is used to improve soil fertility by nitrogen fixation. It is also used in traditional medicine to treat conditions such as burns, wounds, fever, coughs and asthma (Booth et al., 2006).

A lower incidence of cardiovascular diseases (CVDs), cancer and menopausal symptoms is widely reported in Asian women compared to western populations (Yuan, Wang, & Liu, 2007). The Asian diet is rich in soy, and evidence suggests that isoflavones may contribute health beneficial effects (Jerome-Morais, Diamond, & Wright, 2011). Studies on cell cultures, animals and humans have shown that isoflavones exert estrogenic, antioxidant, anti-carcinogenic, antiosteoporotic and antiatherogenic effects (Beck, Rohr, & Jungbauer, 2005).

The biological effects of isoflavones depend on the plasma levels of the compounds, which are highly influenced by their bioavailability. Isoflavones exist as aglycones and glycosides, and in plants isoflavones are mainly found as glycosides which are not readily absorbed due to their hydrophilicity and high molecular weight (Izumi et al., 2000). Post-ingestion aglycones readily penetrate the enterocyte by passive diffusion and are absorbed in the liver, whereas isoflavone glycosides require hydrolysis by glucosidases in the intestine. Glucosidases are mainly produced by intestinal bacteria, where gut microflora have a considerable impact on bioavailability and are thought responsible for the large inter-individual variations (Richelle, Pridmore-Merten, Bodenstab, Enslen, & Offord, 2002;
K. D. Setchell et al., 2001). The absorbed aglycones are either released into the bloodstream in a free active form or undergo conjugation. The first pass enterohepatic conjugation is efficient and isoflavones in plasma are mainly conjugated, either with glucuronic acid or, to a lesser extent, with sulphate (Turner, Thomson, & Shaw, 2003). Urinary and faecal recovery of isoflavones is low, showing that a large proportion of the isoflavones are metabolised. The metabolites are mainly formed in the large intestine and differ from parent molecules in their pharmacological activity (Vitale, Piazza, Melilli, Drago, & Salomone, 2013).

Due to the hydrolytic step required for absorption of isoflavone glycosides, the bioavailability of aglycones is expected to be higher. Isoflavone supplements containing aglycones (30 mg) or glycosides (50 mg) given to healthy men and women showed that aglycones were absorbed faster and in greater amounts (Izumi et al., 2000). A similar result was obtained using a food matrix where isoflavones from fermented soy resulted in a higher bioavailability compared to unfermented soy (Okabe, Shimazu, & Tanimoto, 2011). However, there is also evidence indicating no differences in glycoside and aglycone bioavailability. In one study where 16 women were given tablets with soy isoflavones as aglycone (32 mg) or glycoside (52 mg), only slight differences were seen in maximum concentration and area under the plasma concentration-time curve (AUC). Overall the bioavailability did not differ between the two (Zubik & Meydani, 2003).

These contradictions in results are more likely to be the consequence of heterogeneous study designs (i.e. differences in the ethnicity of subjects, sources and dose of isoflavones, food matrix, duration of study, number of subjects and habitual diet) which confound outcomes when assessing the effect of isoflavone form (aglycone vs. glycoside). Furthermore, large inter-individual differences in the absorption and metabolism of isoflavones by gut microflora present a further challenge to interpreting results.

Bioavailability strongly influences the biological effects of isoflavones, and no clear conclusions can be drawn regarding the impact of a glycoside moiety on bioavailability. As RC extract has been shown to exert beneficial effects on menopausal symptoms and bone health, it is of pharmacological and scientific interest to find the form with the highest bioavailability of isoflavones. It is also of interest to study the bioavailability of isoflavones from fermented RC in different matrices, due to practical issues with the liquid RC extract (unappealing taste, reduced durability, challenges during travels).

With this in mind, the present study aims to test thoroughly the bioavailability of five standardised RC formulations considering diet, food matrix and molecular form. RC formulations were administered in a crossover human study and the pharmacokinetic profiles of the isoflavone formulations were followed for 48 h to investigate the effect of fermentation and matrix on bioavailability.
2 Materials and methods

2.1 Red clover formulations

Five formulations of RC were tested; fermented liquid extract, fermented capsules, fermented tablets, yoghurt with fermented RC and unfermented capsules.

Herrens Mark (Nr. Aaby, Denmark) provided the RC preparations used for the fermented formulations. The production process includes harvesting of the RC plants followed by heating in stainless steel drums. The mass is then filtered and transferred to cooling tanks where lactic acid bacteria is added and the extract is fermented for six months. During the fermentation the pH falls, and the pH of the end product is approximately 4, which acts to preserve the extract. This novel patented fermentation process for RC, gives an extract with a high proportion of aglycone isoflavones. The RC extract was freeze-dried prior to usage in the fermented capsules, tablets and yoghurt formulation.

The fermented capsules and tablets were produced in collaboration with Natur-Drogeriet A/S (Hørning, Denmark). The mixture in the capsules consisted of freeze-dried RC extract, silicon dioxide, magnesium stearate and potato starch. The freeze-dried extract was ground in a blender, and all ingredients were sieved before being mixed manually. The mixture was encapsulated in gelatine capsules using an automated filling machine. The fermented tablets consisted of freeze-dried RC extract, magnesium carbonate and magnesium stearate. The freeze-dried extract was ground in a blender, and all ingredients were sieved before being mixed in an automated mixer and compressed to tablets. The yoghurt formulation was prepared by the project team as a mixture of skyr (Levevis, Dansk Supermarked A/S, Højbjerg, Denmark), freeze dried RC extract, blueberries (Vores, Dansk Supermarked A/S, Højbjerg, Denmark), stevia powder (steviol-glycosides, Steviafarma Industrial S/A, Maringá, Brazil) and almond essence (Dr. Oetker, Glostrup, Denmark). The unfermented capsules are a commercial RC supplement containing 400 mg pulverised RC per capsule (Jordens Frugter, Aarhus, Denmark).

2.2 HPLC analysis of isoflavones in red clover formulations

Quantification of isoflavones in the RC formulations was performed by DB lab (DB Lab A/S, Odense, Denmark) utilising high performance liquid chromatography (HPLC) with UV detection. Analysis was carried out pre- and post-acidic hydrolysis to determine aglycone content.

The samples were prepared for analysis in the following ways. 10 g RC extract was mixed with 50 mL methanol (VWR chemical, Søborg, Denmark) and filtered through a 0.45 μm nylon filter (Q-Max, Frisenette, Knebel, Denmark). Acidic hydrolysis of RC extract was carried out by mixing 20 mL RC extract, 8.3 mL 37% hydrochloric acid (Merck KGaA, Darmstadt, Germany) and 71.7 mL
ethanol (Merck KGaA, Darmstadt, Germany). The mixture was heated to 80 °C for 5 hours. 400 mg of freeze-dried RC extract was mixed with appr. 80 mL ethanol and treated in ultrasonic bath for 40 minutes. The mixture was topped with ethanol until 100 mL and filtered through a 0.45 µm nylon filter. Acidic hydrolysis of freeze-dried RC extract was carried out by mixing 400 mg freeze-dried RC extract with 60 mL ethanol and 20 mL 5M hydrochloric acid. The mixture was heated to 80 °C for 5 hours, then topped with ethanol until 100 mL and filtered through a 0.45 µm nylon filter. The powder from the unfermented capsules was treated in the same way as the freeze-dried RC extract.

The HPLC system was a Summit 4 (Thermo Scientific, San Jose, CA) consisting of a P680 LPG pump, ASI 100T autosampler, TCC-100 column oven and PDA-100 detector. It was equipped with a Polaris 3 C18-A column (250 x 3 mm, 3 µm particle size, Agilent technologies, Glostrup, Denmark). The mobile phase consisted of 10% 0,1% formic acid (Sigma-Aldrich, Brøndby, Denmark) in a mixture of water (A) and acetonitrile (B) (VWR chemical, Søborg, Denmark) as follows; 0 to 57 min 18% B, 57 to 62 min 90% B, 62 to 70 min 18% B. The flow rate was 0,4 mL/min and the injection volume was 10 µL. The isoflavones were quantified by UV detection at 260 nm.

### 2.3 Subjects and study design

The study protocol was approved by the Local research ethics committee in region Central Jutland (Denmark) and the trial registered at ClinicalTrials.gov (NCT02264223). Written informed consent was obtained from all participants.

A power calculation using a least significant difference of 8% and values of AUC from a study by Okabe as a guideline showed that 13 participants were necessary. In order to consider the usual percentage dropout, 15 participants were recruited.(Okabe et al., 2011)

A randomized crossover study of 15 healthy women aged 19-39 years was performed. Exclusion criteria included current hormone treatment, pregnancy, lactation, use of medications that affect uptake (except contraceptives), drug or alcohol abuse, cardiovascular, psychiatric, neurological or renal disorders and acute diseases. During the screening process health status was evaluated by measuring blood pressure and markers of liver and kidney function.

The participants received all five formulations and are used as their own controls. The participants received RC formulations in random order, and each intervention was separated by wash-out periods of a minimum of 5 days. The participants followed an isoflavone-free diet one week before trial start and during the whole duration of the trial.

After an overnight fast, the RC formulations were provided immediately prior to breakfast. Participants received standardized meals on the intervention day and the following day, which were prepared by members of the project team. On the intervention day lunch was given at noon after blood was drawn at 4 h and dinner was given at 18:00 approximately 10 h after RC administration. The
nutritional composition of the standardised diet followed the nutritional recommendations for a healthy diet and the mean energy content was 9075 kJ/day. The percentage distribution of macronutrients was 17.5 % protein, 52.5 % carbohydrates and 30.5 % fat (for details on the diet see table A.1 in Appendix A). Diet diaries for the day before each intervention and the two intervention days were filled out. Blood samples were collected before consumption (baseline) of the RC and 2, 4, 6, 8, 12, 24 and 48 h after intake. EDTA plasma was centrifuged (3000 x g, 10 min, 20 °C) and stored at -80 °C until further analysis.

To obtain the 40 mg isoflavones aglycone equivalents required in the study, the participants were administered 61.7 mL RC extract, 2.86 g freeze-dried RC extract or fifteen 500 mg unfermented capsules.

2.4 Extraction of isoflavones in plasma

Plasma samples were pretreated with β-glucuronidase from Helix Pomatia (type HP-2, > 100,000 U/mL, Sigma-Aldrich, Brøndby, Denmark). 1 mL plasma was incubated with 120 µL β-glucuronidase and 1010 µL sodium acetate buffer (0.945 g sodium acetate powder (VWR chemical, Søborg, Denmark), 172.5 µL acetic acid (glacial) (Merck KGaA, Darmstadt, Germany), 50 mL pure water) for 1 hour at 45 °C. The plasma samples were extracted by mixing the hydrolysed plasma mixture with 3 mL acetonitrile (HPLC purity > 98%, Sigma-Aldrich, Brøndby, Denmark). The mixture was vortexed for 30 s every 5 min for 3 times and centrifuged at 190 x g at 20 °C for 10 min. The supernatant was collected, filtered through a 0.22 µm filter (Q-Max, Frisenette, Knebel, Denmark) and evaporated to dryness at 34 °C in a speed vacuum centrifuge (Scan Speed 40, Scanvac, Lyngby, Denmark). The isoflavones were reconstituted in 400 µL of mobile phase (0.1% formic acid (HPLC purity > 98%, Sigma-Aldrich, Brøndby, Denmark) in 60% acetonitrile and 40% water) and 50 µL ethanol (HPLC purity > 98%, Sigma-Aldrich, Brøndby, Denmark).

2.5 LC-MS analysis of isoflavones in plasma

The plasma samples were analysed for daidzein, genistein, formononetin and biochanin A content by LC-MS. The LC-MS consisted of a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) and an HPLC system with an Accella 1250 pump and a CTC PAL autosampler (Thermo Scientific, San Jose, CA). The Mass spectrometer was fitted with an ESI source and run in SRM mode with a collision gas pressure of 1.5 mTorr and with the following tune settings. The capillary and vaporizer temperatures were 200 °C and 350 °C respectively, the sheath gas and ion sweep gas pressures were 40 and 0 respectively, the auxiliary gas flow was 20 L/min and the spray voltage was 3000 V in negative mode. The parent ions, product ions and collision energies for each compound are given in Table 1.
Table 1: Parent ions, product ions and collision energies on MS.

<table>
<thead>
<tr>
<th></th>
<th>Parent ion</th>
<th>Product ion</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzein</td>
<td>253</td>
<td>224</td>
<td>28</td>
</tr>
<tr>
<td>Genistein</td>
<td>269</td>
<td>133</td>
<td>33</td>
</tr>
<tr>
<td>Formononetin</td>
<td>267</td>
<td>252</td>
<td>22</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>283</td>
<td>239</td>
<td>34</td>
</tr>
</tbody>
</table>

The HPLC column was a Kinetex 2.6 µm, C18, 100 Å, 150 x 4.6 mm (Phenomenex, Værløse, Denmark). The column was kept at a constant temperature of 30 °C and the injection volume of sample was 10 µl. Elution was performed with a flow of 200 µL/min with ultrapure water with 0.1% formic acid (HPLC purity > 98%, Sigma-Aldrich, Brøndby, Denmark) (solvent A) and acetonitrile (HPLC purity > 98%, Sigma-Aldrich, Brøndby, Denmark) with 0.1% formic acid (solvent B) with the following gradient programme; 0 to 1 min isocratic 15% solvent B, 1 to 20 min linear gradient from 15 to 25% solvent B, 20 to 25 min isocratic 25% solvent B, 25 to 40 min linear gradient from 25 to 60% solvent, 40 to 55 min linear gradient from 60 to 99% solvent B, 55 to 60 min isocratic 99% solvent B, 60 to 62 min linear gradient from 99 to 15% solvent B and 62 to 72 min isocratic elution with 15% solvent B.

2.6 Statistical analyses

Means and standard deviations were calculated for demographic data and diet registrations. Statistical differences between the macronutrient compositions of the diet for the five interventions were analysed by one-way ANOVA with a Bonferroni post-test. Differences were considered significant when p < 0.05.

The bioavailability was evaluated from the incremental area under the concentration-time curve (iAUC), which was calculated using the trapezoidal rule using the 0 hour plasma concentration as baseline value. Results are given as means ± SEM. Statistical differences between the bioavailability of formulations were evaluated with a paired student’s t test. Differences were considered significant when p < 0.05.

iAUC calculation and statistical analyses were carried out using GraphPad Prism version 4.03 for Windows.

3 Results

3.1 Isoflavones in red clover formulations

The concentrations of isoflavones in the RC formulations were determined pre- and post-acidic hydrolysis, and the results for the hydrolysed samples for the four main isoflavones are shown in
Table 2. The values were used to calculate the dosage of the formulations to obtain 40 mg total isoflavones, and the intake of the individual isoflavones per dose are also given in the table. The isoflavones glycitein and ononin were found in minor amounts.

Table 2: Isoflavone concentrations in RC after acidic hydrolysis (aglycone equivalents) and intake of isoflavones at dosage of 40 mg total isoflavones.

<table>
<thead>
<tr>
<th></th>
<th>Fermented extract</th>
<th>Freeze-dried fermented extract</th>
<th>Unfermented capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. (mg/L)</td>
<td>Intake per dose (mg)</td>
<td>Conc. (mg/kg)</td>
</tr>
<tr>
<td>Daidzein</td>
<td>31</td>
<td>1.9</td>
<td>164</td>
</tr>
<tr>
<td>Genistein</td>
<td>54</td>
<td>3.3</td>
<td>1286</td>
</tr>
<tr>
<td>Formononetin</td>
<td>342</td>
<td>21.1</td>
<td>7842</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>221</td>
<td>13.7</td>
<td>4690</td>
</tr>
<tr>
<td>Total</td>
<td>648</td>
<td>40</td>
<td>13982</td>
</tr>
</tbody>
</table>

The distribution of isoflavones in the unfermented capsule is very different from the fermented formulations. The percentage content of formononetin is considerably lower in the unfermented capsule (27 % vs. 54 %), and the content of the three other isoflavones are higher (22 % vs. 8 % for genistein, 7 % vs. 3 % for daidzein, 44 % vs. 34% for biochanin A). It is therefore necessary to look at the iAUCs for all four isoflavones when comparing the bioavailability of different formulations.

The fermentation process changes the ratio between aglycones and glycosides, and the aglycone content in the fermented RC formulations is approximately twice as high as the unfermented RC (Table 3).

Table 3: Dose of RC formulations to obtain 40 mg isoflavone aglycone equivalents and distribution of isoflavone forms.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Dose</th>
<th>Aglycone (%)</th>
<th>Glycoside (%)</th>
<th>D+F/G+B ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermented capsule</td>
<td>6 pcs.</td>
<td>71</td>
<td>29</td>
<td>1.34</td>
</tr>
<tr>
<td>Unfermented capsule</td>
<td>15 pcs.</td>
<td>35</td>
<td>65</td>
<td>0.52</td>
</tr>
<tr>
<td>Liquid extract</td>
<td>61.7 mL</td>
<td>76</td>
<td>24</td>
<td>1.36</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>2.86 g ‡</td>
<td>71</td>
<td>29</td>
<td>1.34</td>
</tr>
<tr>
<td>Fermented tablet</td>
<td>4 pcs.</td>
<td>71</td>
<td>29</td>
<td>1.34</td>
</tr>
</tbody>
</table>

† Ratio of daidzein and formononetin over genistein and biochanin A.
‡ Amount of freeze dried RC extract mixed into yoghurt formulation.

3.2 Demographic data and diet

Fifteen healthy women were recruited and completed the five interventions. The mean age of the participants was 25 ± 6 years. Data from pre-study screening are given in Table 4.
The diets on the two days of intervention were standardized and macronutrient compositions of the diets during the five interventions are shown in Table 5. These were assessed through diet diaries and no statistical differences were observed between intakes of the five formulations.

Table 5: Macronutrient composition of diets (mean of the two intervention days) (means ± SD, n=15).

<table>
<thead>
<tr>
<th></th>
<th>Energy (kJ/d)</th>
<th>Protein (g/d)</th>
<th>Fat (g/d)</th>
<th>Carbohydrate (g/d)</th>
<th>Fibre (g/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermented capsule</td>
<td>8981 ± 1052</td>
<td>89 ± 10</td>
<td>70 ± 12</td>
<td>285 ± 50</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Unfermented capsule</td>
<td>8463 ± 1062</td>
<td>84 ± 12</td>
<td>67 ± 8</td>
<td>274 ± 47</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Liquid extract</td>
<td>8885 ± 1048</td>
<td>88 ± 8</td>
<td>69 ± 11</td>
<td>287 ± 42</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>8823 ± 861</td>
<td>89 ± 7</td>
<td>68 ± 13</td>
<td>283 ± 39</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Fermented tablet</td>
<td>8582 ± 791</td>
<td>89 ± 7</td>
<td>66 ± 9</td>
<td>277 ± 37</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>P value</td>
<td>&gt; 0.05</td>
<td>&gt; 0.05</td>
<td>&gt; 0.05</td>
<td>&gt; 0.05</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

3.3 Plasma concentrations of isoflavones

Due to challenges with the highly sensitive analytical equipment, it has only been possible to determine the isoflavone content in approximately 10% of the collected plasma samples within the given time frame of the project. We have chosen to analyse samples taken at 0, 2, 6 and 24 hours for five participants and three formulations (liquid extract (lex), fermented capsule (fcap) and unfermented capsule (ufcap)).

Figure 1 shows plasma concentration curves for total isoflavones (sum of daidzein, genistein, formononetin and biochanin A) for the three formulations.
Before ingestion the total isoflavone concentrations are 0.15 - 0.18 µmol/L for the three formulations. Peak concentrations of total isoflavones were reached after 6 hours for liquid extract and unfermented capsule, and reached maximum concentrations of 0.24 and 0.23 µmol/L respectively. There was no clear peak concentration for the fermented capsule. Twenty-four hours after the ingestion of RC the total isoflavone plasma concentration fell, but did not reach equivalence to baseline value. Similar patterns are seen for the individual isoflavones (daidzein, genistein, formononetin) with peak plasma concentrations being reached after 6 hours (data not shown). The relationship between formulation and maximum concentrations are however different for the individual isoflavones which is reflected in the iAUCs shown in Table 6. When looking at daidzein and formononetin the highest iAUC is obtained in the fermented formulations, whereas for genistein the highest iAUC is seen for the unfermented capsule.

Table 6: Values for iAUC<sub>0-24h</sub> for plasma isoflavones after ingestion of RC formulations. Total isoflavones were calculated as the sum of daidzein, genistein, formononetin and biochanin A. (means ± SEM, n=5)

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>Liquid extract</th>
<th>Fermented capsule</th>
<th>Unfermented capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1.04 ± 0.15</td>
<td>1.21 ± 0.28</td>
<td>0.99 ± 0.12</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.62 ± 0.07</td>
<td>0.58 ± 0.12</td>
<td>0.27 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.33 ± 0.05</td>
<td>0.35 ± 0.07</td>
<td>0.61 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formononetin</td>
<td>0.09 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Biochanin A †</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>†</sup> Values for biochanin A are not shown as the plasma concentration changes are very low and it is not possible to calculate the area under the concentration curve.
<sup>a</sup> Significantly different from liquid extract (p < 0.05).
<sup>b</sup> Significantly different from liquid extract and fermented capsule (p < 0.005).

The mean values of iAUC for total isoflavones for the three formulations are shown in Figure 2. There is a tendency that total bioavailability of isoflavones is higher for the fermented capsules compared...
with the unfermented, however no statistical differences can be seen between the total isoflavone iAUCs for the three formulations.

![Graph showing total isoflavones](image)

*Figure 2: iAUC 0-24h for liquid extract (lex), fermented capsule (fcap) and unfermented capsule (ufcap) for total isoflavones. Paired t-test shows no significant differences. Data are shown as means ± SEM.*

When looking at daidzein and genistein separately statistical differences were obtained (Figure 3). For daidzein, iAUC was significantly higher for the liquid extract compared to unfermented capsules. For genistein, iAUC for the unfermented capsules was significantly higher compared to the two fermented formulations.

![Graph showing daidzein and genistein](image)

*Figure 3: iAUC 0-24h for liquid extract (lex), fermented capsule (fcap) and unfermented capsule (ufcap) for daidzein and genistein. *P < 0.05, **P < 0.005. Data are shown as means ± SEM.*

There were large variations in changes in plasma concentrations between the subjects, especially in the initial increase after 2 hours.
4 Discussion

Enhancing the bioavailability of isoflavones increases their biological activity. Physiologically relevant plasma concentrations are needed to exert health beneficial effects. Hence, it is important to take bioavailability into account when investigating potential health effects of isoflavones. Bioavailability of isoflavones depends on many factors including isoflavone source and form, food matrix and habitual diet. Many studies have been carried out to investigate the impact and magnitude of these factors on bioavailability. Studies have mainly shown that aglycones have higher bioavailability than glycosides, which is due to their more lipophilic character and lower molecular weight (Izumi et al., 2000; Okabe et al., 2011). A few studies have failed to show differences between the two forms or even find that glycosides are more bioavailable (K. D. Setchell et al., 2001; Zubik & Meydani, 2003). Studies on the effect of food matrix have demonstrated that there is a tendency for higher bioavailability for liquids compared to solids (Cassidy et al., 2006). Studies on bioavailability are difficult to compare due to heterogeneity between study designs (study duration, isoflavone source, dose etc.) and extensive differences in inter-individual metabolism. Variations in extraction and analytical methods used further contribute to disparities apparent in plasma concentrations between studies.

We found that all four isoflavones of interest (daidzein, genistein, formononetin and biochanin A) could be detected in the plasma samples, and that daidzein represented the highest concentrations followed by genistein and formononetin. Biochanin A was detected in negligible amounts. Approximately the same pattern was observed in a study by Maul et al. where a RC supplement with similar composition as ours was ingested. They found daidzein in highest concentrations followed by formononetin and genistein (Maul & Kulling, 2010). It is however contradictory to studies using soy, where plasma concentrations of genistein have generally been higher than daidzein despite similar intakes, but this may be due to differences in isoflavone composition in the source (Izumi et al., 2000; Watanabe et al., 1998). The iAUC for total isoflavones was 1.04 ± 0.15, 1.21 ± 0.28 and 0.99 ± 0.12 µmol·24h/L for liquid extract, fermented capsule and unfermented capsule respectively. There is no statistical significant difference between the values for the three formulations but there is a tendency for a higher bioavailability for the fermented formulations.

Looking at the isoflavones separately different patterns emerge. In the case of daidzein the largest plasma concentrations and iAUC is seen for the liquid extract, which is also expected as the isoflavones are mainly found in aglycone form and they are in a liquid matrix. The iAUC is significantly higher in the extract compared to the unfermented capsules. For formononetin there was a tendency for higher iAUC for the fermented formulations compared to the unfermented formulation, but differences did not reach statistical significance. However, when looking at genistein, the unfermented capsule shows a significantly higher iAUC than the two fermented formulations. This is unexpected due to isoflavones found mainly as glycosides. There is however, a difference in the ratios of daidzein and formononetin over genistein and biochanin A in the fermented and unfermented formulations (Table 3). The ratio for the fermented formulations is on average 1.35 whereas the ratio is 0.52 for the unfermented capsule. This should be taken into consideration and the most reliable
picture regarding bioavailability in general is obtained when looking at total isoflavone concentrations. There are no significant differences in bioavailability between the fermented liquid extract and fermented capsules either when looking at total or the individual isoflavones.

When investigating the effect of isoflavone form and matrix on bioavailability differences in isoflavone source can be a challenge. In this study the isoflavones in the fermented liquid, tablet, capsule and yoghurt formulation stem from the same source, this is advantageous as it strengthens the ability to study the effect matrix on bioavailability. There are slight differences in the percentage distribution of the four studied isoflavones between the liquid extract and the freeze-dried extract but these are considered to negligibly influence the results. The cause for these slight differences stems from the different batches of RC used for the liquid and freeze-dried extract. As the isoflavone content depends on environmental conditions and varies between harvests, it is natural that variations in content and distribution are seen. However, a clear difference is apparent when comparing the distributions for the fermented and unfermented formulations. The content of daidzein and its methylated form formononetin is 1.3 times higher in the fermented formulations than in the unfermented formulation, whereas the concentration of genistein and its methylated form biochanin A is almost doubled in the unfermented formulation compared to the fermented formulations.

It is unknown why these differences are seen, especially for formononetin, which is expected to represent the highest concentrations in RC preparations. During the analyses of the formulations, the hydrolysed samples were not analysed for remaining glycosides and it is unknown whether a full hydrolysis was achieved. If this is not the case, the deviation will be most pronounced for the unfermented formulation containing the highest concentration of glycosides. Another factor contributing to the lower isoflavone concentrations found in the unfermented capsules may be the presence of fibre material from the RC plant, as this formulation consists solely of pulverised RC. In contrast, the fermented RC is a more pure formulation devoid of plant material. These differences in isoflavone distribution may affect bioavailability and confound results, and are probably the main reason why different patterns are seen when considering daidzein and genistein separately. It could have been an advantage to test an unfermented formulation from the same RC source as the fermented formulation; unfortunately, this was not possible in the study. The variations in isoflavone distribution seen in the fermented and unfermented RC formulation represents the general pattern of the market for supplements, where a wide inter- and intra-product variation in the composition is often present and little standardisation takes place (K. D. Setchell et al., 2001). As isoflavones are shown to have different biological activities, it is of utmost importance to consider the isoflavone source when studying physiological effects.

The low concentrations of isoflavones detected in the unfermented capsules used required fifteen capsules to be taken to obtain the equivalent dose of 40 mg total isoflavones (the chosen dose resembles average daily intakes of Asian populations (Messina, Nagata, & Wu, 2006)). Fifteen is an unrealistic amount to take on a daily basis to relieve menopausal symptoms or prevent bone demineralisation. Hence development of fermented RC formulations with higher and standardised isoflavone concentrations would provide a clearly preferable solution.
We initially attempted to use quadropole time-of-flight mass spectrometer (QTOF-MS) to quantify isoflavones (aglycones and metabolites) in plasma, as this has not been done in previous studies and would give information on isoflavone metabolism. However, we did not manage to measure and quantify the isoflavones with this method. Due to these and other challenges with analytical equipment and time constraints during the analysis stage of the trial, it was not possible to analyse all the collected plasma samples. Therefore, the concentration time curves are only based on four measurements. This increases the risk of skewness of data curves leading to less reliable data and increased false negative/false positive outcomes. The prioritised time points have been chosen by looking at results from other bioavailability studies, and consideration must be given the missing measurements, particularly when determining the maximal concentration point. In other studies the concentration time data shows that there is a steady decline between 8 and 24 hours post intake, this implies that our graph closely resembles reality. In the early hours after ingestion our graphs may be less reliable and representative, especially for the fermented formulation, as maximum concentrations are expected to be reached at an earlier time. This has been shown in a study with soy powder where maximum concentration was reached 1-2 hours after ingestion for the aglycone-rich powder whereas for the glycoside-rich formulation maximum concentration was obtained after 4-5 hours (Okabe et al., 2011). Our results show peak concentrations at 6 hours for all formulations. Remaining samples (five for each intervention) will be analysed later, and when values at 4 and 8 hours are available, differences in time to reach maximum concentration may be observed. There are large variations in the absorption patterns between the subjects, and some of the graphs have shapes that are difficult to explain. This may be due to the few points on the curve; once data from all time points are available, a clearer picture of the absorption and excretion patterns will be obtainable.

The isoflavone concentrations in our samples are lower than expected. In a study by Maul et al. subjects ingested 38.8 mg RC isoflavones as capsules and plasma samples were taken after 6.5 hours (Maul & Kulling, 2010). The dose is close to the dose in the present study, and even though the isoflavone composition was different, the daidzein and formononetin content was similar. Despite of this, plasma concentrations of these two isoflavones were considerably higher in the female participants in their study (0.297 µM and 0.082 µM for daidzein and formononetin respectively) compared to our values for the 6 hour samples (0.097 µM and 0.048 µM for daidzein and formononetin respectively). de Pascual-Teresa measured isoflavone uptake after ingestion of 50 mg soy isoflavones incorporated in food matrices. Maximum plasma concentrations were 0.4 – 0.6 µM which are approximately five times higher than the concentrations measured in this study (de Pascual-Teresa et al., 2006). The low plasma concentrations we measured are unexpected as the isoflavone dose was similar in the studies and the participants were healthy and of European origin. The average isoflavone intake in European countries is 1.4 mg/day, so when an isoflavone dose approximately thirty times higher was administered, we expected higher plasma isoflavone concentrations than twice the baseline value (Zamora-Ros et al., 2012).

A possible explanation could be that the extraction method requires optimisation and only a small percentage of the isoflavones are extracted from the plasma. Several methods have been used for
sample preparation when analysing isoflavones in plasma, and this can contribute to observed
differences between studies. In the current study, the extraction method used was based on
experiences with isoflavone analyses and similar to methods in other studies. Time did however not
allow for validation of the method, and the method might not be optimal for this purpose, as the use
of other extraction solvents and an internal standard might be appropriate. Furthermore, the hydrolysis
time was based on results from a study by Taylor et al. showing that daidzein and genistein were
hydrolysed after 40 minutes, but a longer hydrolysis time may be necessary to reach higher
concentrations of the aglycones (Taylor, Grace, & Bingham, 2005). This is especially important when
using a triple quadropole MS where you specify the molecules of interest. With incomplete hydrolysis
there is potential for missed intact glucuronides and sulphated conjugates. Due to their hydrophobic
nature, aglycones may bind to plastic surfaces they come in contact with during sample preparation,
which can lead to significant losses of isoflavones (Griffith & Collison, 2001). It may therefore be
more appropriate to use glass utensils where possible, especially for the steps where the sample is in
contact with the surface for a longer time. As the utensils used are seldom mentioned in literature,
focus has not been put on this during our sample preparations, and time did not allow for recovery
studies, which could have clarified whether significant losses took place. The low plasma
concentrations make the results more vulnerable to variations in measurements, and several factors
in our sample preparation may contribute to these low concentrations. However, as it is a cross-over
study and all samples were treated in the same way, it is estimated that even though the extraction
method does not extract all the isoflavones, comparison of the formulations are still possible as they
have been exposed to the same magnitude of error.

The participants were asked to avoid isoflavone containing foods in their diet one week prior to the
study and for the whole duration of the study. We expected the baseline concentrations to be close to
zero, and were surprised to find concentrations, which were high compared to the measurements at
later time points and values found in other studies. The mean baseline values for daidzein and
genistein were 0.07 and 0.04 µM respectively, and are at least ten times higher than values found for
the Danish participants in the European Prospective Investigation into Cancer and Nutrition (EPIC).
They found mean plasma concentrations of daidzein and genistein of 0.004 µM (Peeters et al., 2007).
The reason for this difference is unknown but variations caused by the sample preparation method
mentioned previously might contribute. Consequently, the iAUCs have been used taking baseline
values into account.

Isoflavones in liquid formulations might be more bioavailable as they are already in solution and the
solubility affects the rate of absorption. One study has shown higher AUC and maximal
concentrations of isoflavones when ingesting soy milk compared to solid textured vegetable protein
(TVP) (Cassidy et al., 2006). However, other studies have failed to show a clear effect as differences
between absorption of isoflavones from soy milk and TVP were only observed for genistein and only
in women (Faughnan et al., 2004). Other factors than food matrix may affect the bioavailability
including isoflavone composition as this will be different in soy milk and TVP even though they are
both from soy. In this study the liquid and solid (capsule) formulations have approximately the same
isoflavone composition and no significant differences in bioavailability were seen. This evaluation is
however based on a limited number of analysed samples and low plasma concentrations, and patterns may change when all results are available. It would be beneficial if bioavailability is preserved after processing the extract to a capsule, as capsules would solve a number of issues regarding palatability, storage and logistical challenges of the extract.

Studies have shown that the bioavailability of isoflavones is higher for aglycones than for glycosides as the initial hydrolysis step is not needed. Okabe et al. found an increase of 60% and 20% of the maximal plasma concentration and the AUC respectively, when ingesting an aglycone-rich soybean formulation compared with a formulation rich in glycosides (Okabe et al., 2011). One method of converting glycosides into aglycones is by fermentation with lactic acid bacteria. This process doubled the amount of aglycones in the fermented RC formulations in contrast to the unfermented formulation in this study (Table 2). We failed to show the expected difference between fermented and unfermented formulations in terms of total isoflavone uptake. There is a tendency for a higher bioavailability of the fermented formulations when looking at Figure 2, however the differences are small and were non-significant. As the results are at present only based on five participants, the statistical foundation is weak and it is expected that the patterns will become more clear and robust when samples from all 15 participants and all data points have been analysed.

The isoflavone content in RC is unique with a high content of formononetin and biochanin A compared to soy. This has an influence on its biological effects as formononetin has been shown to have an increased potency on osteoblast function compared to daidzein (Gautam et al., 2011). However, a great conversion of formononetin and biochanin A into their demethylated forms daidzein and genistein takes place, and lower concentrations of formononetin and biochanin A are often found in plasma compared to daidzein and genistein even when administered in higher doses. Few studies, with a limited number of participants, have looked at plasma concentrations of formononetin and biochanin A after red clover intake. They found that substantial demethylation takes place, and even though the intakes of the methylated isoflavones are 10 - 40 times higher than the demethylated isoflavones, the ratio of methylated over demethylated isoflavone ranges from 0.2 - 0.5 in plasma (Howes et al., 2000; Maul & Kulling, 2010; K. D. Setchell et al., 2001). We expected to see increases in concentrations of formononetin and biochanin A based on the findings of these studies and due to the high contents in the RC formulations. Surprisingly we found no increases in biochanin A concentrations and the increases for formononetin are relatively minor. Issues with the sample preparation, as mentioned earlier, might explain this. Despite the low concentrations, an evaluation of the iAUC for formononetin shows a tendency for a higher bioavailability for the fermented formulations, which could be a reflection of the higher concentration of formononetin in these formulations.

Equol is a metabolite of daidzein that has higher estrogenic activity than daidzein and therefore potentially exerts greater biological effects than its parent molecule. Equol is produced in the large intestine by gut microbiota but not all people are able to produce equol. Appr. 30% of the American and European population have this ability whereas in Asia the proportion is appr. 50% (Okabe et al., 2011). Some of the participants in this study may be equol producers, which would lead to lower
plasma concentrations of daidzein. Plasma concentrations of equol were not measured in this study, but would have given a fuller picture of the total amount of isoflavones being absorbed. However, the main purpose of this study is to compare the bioavailability of different formulations. As it is a cross-over study the subjects act as their own controls and it can be assumed that the subjects capable of equol production produce consistently across all five formulations.

Diet may have an effect on isoflavone pharmacokinetics, as it affects gut microflora and thereby metabolism and absorption. In this study standardised diets were given to the participants during the interventions, and no differences in energy or macronutrient intake were seen between the five interventions (Table 5). The diet on the day before the intervention was not standardised but registered in diet diaries. Large variations were seen between the participants and between the interventions; as such it might have been advantageous to extend the standardised diets to start prior to the intervention day. Registration of diet the day prior to ingestion of a formulation reflects habitual diets of the participants, this may have a greater influence on bioavailability than the post ingestion diet given during the study. These large differences in habitual diet may partly explain the large inter-individual differences in bioavailability. Gut transition time and genetics also have an impact on absorption and metabolism of isoflavones and contribute to inter-individual variations, but as this is a cross-over study the influence of this variation is reduced.

The standardised diet followed nutritional recommendations for a healthy lifestyle and was therefore higher in dietary fibres than the diets registered on the days prior to formulation intake, and the habitual diets of a great proportion of the Danish population. It has been shown that dietary fibre has an impact on bioavailability, some studies have shown increased bioavailability after addition of inulin while others show reduced absorption with increasing fibre content in the diet (Piazza et al., 2007; Tew, Xu, Wang, Murphy, & Hendrich, 1996). The direct effect of fibres in the diet on bioavailability is therefore unclear, and it is uncertain whether it is a long term effect or whether the fibre content in the diet has a more immediate impact when ingested simultaneously with isoflavones. It is a possibility that the relatively high fibre content in the standardised diet contributes to the low isoflavone plasma concentrations seen in this study.

Bioavailability of isoflavones may be influenced by the context in which the isoflavones are ingested, especially in the case of isoflavones taken as supplements. An in vitro study by Walsh et al. showed that concentrations of bile affected the bioavailability of isoflavones, it is proposed that isoflavones in a food matrix or eaten together with foods containing fat and protein have higher bioavailability than isoflavones from supplements taken without a meal (Walsh, Zhang, Vodovotz, Schwartz, & Failla, 2003). In the current study the RC formulations were provided together with a breakfast meal, which should even out variations caused by differences in bile secretion.

Lactic acid bacteria are used as probiotics as they influence gut microflora and they may therefore affect bioavailability of isoflavones. The studies in this field show mixed effects of probiotics on bioavailability, which might primarily be due to differences in dosage levels and duration of intake (Cohen et al., 2007; Larkin, Price, & Astheimer, 2007; Nettleton et al., 2004). In order for probiotics
to have a beneficial impact on gut microflora long-term intake is probably necessary. Lactic acid bacteria are used in the fermentation process for four RC preparations used in the present study. The bacteria found in the freeze-dried extract are in lower concentrations due to losses during processing. The presence of lactic acid bacteria in these RC formulations might contribute to increased absorption of isoflavones after long-term intake.

In the current study, young healthy women were chosen as subjects in order to reduce variations in health status. However, the main target group for RC products is menopausal and postmenopausal women experiencing hot flashes and bone demineralisation. It is unknown whether the absorption patterns would have been different for these women. A study of the effect of age on urinary excretion of isoflavones failed to show a difference between pre- and postmenopausal women (Faughnan et al., 2004). Another study on plasma also failed to show a significant effect of age and menopausal stage on the bioavailability of isoflavones (K. D. R. Setchell et al., 2003). It is therefore assumable that the absorption patterns observed in this study will be similar in postmenopausal women.

Because bioavailability is important, methods of increasing absorption are of interest. Apart from the potential factors mentioned previously (fermentation, probiotics) microencapsulation may be a useful method. Microencapsulation is used in the food and pharmaceutical industry and could increase bioavailability of isoflavones by increasing residence time and obtaining more constant plasma concentrations (K. D. R. Setchell et al., 2005). Future studies of the effects of this method as well as large long-term studies of the influence of isoflavone form, matrix, dose etc. on bioavailability will be useful in determining the optimal dosage and form of isoflavone intake to obtain desired physiological effects.

We attempted to investigate the bioavailability of isoflavones from fermented and unfermented RC formulations (five in total) by analysing blood samples from fifteen healthy young women recruited for this cross-over study. Due to considerable challenges with analytical equipment, it was only possible to analyse approximately 10% of all samples within the time frame of this project, and interpretations are based on this reduced number of samples. The results suggest a tendency for total isoflavone bioavailability being higher for fermented RC (liquid extract and capsules) compared to unfermented capsules, even though the results did not reach statistical significance. When looking at daidzein there was a significantly higher bioavailability for the fermented extract compared to the unfermented capsule. The total isoflavone bioavailability was almost equal in the fermented liquid extract and capsules, indicating that processing does not reduce isoflavone bioavailability. Stronger conclusions can be drawn when data from all samples are available.
Conflict of interest

The authors declare that they have no conflicts of interests.

Acknowledgements

The author wishes to thank Michael Mohr Jensen (Herrens Mark) for providing RC extract and Hans-Christian Vollstedt (Naturdrogeriet) for production of the fermented capsules and tablets. Also a thank you to Lars Jørgensen (DBLab), Xavier Fretté, Flemming Nielsen and Rime Bahij El-Houri (University of Southern Denmark) for analytical assistance, and to the women who participated in the study.

References


Griffith, a P., & Collison, M. W. (2001). Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-


### Appendix A

Table A.1: Standardised diet during intervention

<table>
<thead>
<tr>
<th>Day</th>
<th>Meal</th>
<th>Menu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Breakfast</td>
<td>1 bread roll, 2 slices of cheese, butter, marmalade, orange- or apple juice, 1 apple, coffee or tea</td>
</tr>
<tr>
<td>1</td>
<td>Lunch</td>
<td>Sandwich with chicken, tomato, cucumber, lettuce and mayonnaise</td>
</tr>
<tr>
<td>1</td>
<td>Dinner</td>
<td>Pasta with leeks, ham, cheese, cream and eggs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed salad (carrot, pointed cabbage, chinese cabbage, corn, tomatoes)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salad dressing</td>
</tr>
<tr>
<td>1</td>
<td>Snacks</td>
<td>2 carrots, 1 bread roll, butter, 1 banana, 1 pear, 1 muesli bar with chocolate</td>
</tr>
<tr>
<td>2</td>
<td>Breakfast</td>
<td>1 bread roll, 2 slices of cheese, butter, marmalade, orange- or apple juice, 1 apple, coffee or tea</td>
</tr>
<tr>
<td>2</td>
<td>Lunch</td>
<td>2 pita breads with ham, tuna, mixed salad (carrot, pointed cabbage, chinese cabbage, corn, tomatoes) and salad dressing</td>
</tr>
<tr>
<td>2</td>
<td>Dinner</td>
<td>Lasagne with chicken, tomato, onion, garlic, carrots, courgettes and mushrooms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed salad (carrot, pointed cabbage, chinese cabbage, corn, tomatoes)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salad dressing</td>
</tr>
<tr>
<td>2</td>
<td>Snacks</td>
<td>2 carrots, 1 bread roll, butter, 1 banana, 1 pear, 1 muesli bar with nuts</td>
</tr>
<tr>
<td>3</td>
<td>Breakfast</td>
<td>1 bread roll, 2 slices of cheese, butter, marmalade, orange- or apple juice, 1 apple, coffee or tea</td>
</tr>
</tbody>
</table>