

Phytoestrogens from soy and red clover preparations - well characterized dietary supplements?

R. Maul¹; J.F. Wollenweber²; S.E. Kulling³

¹ University of Hamburg, Institute of Biochemistry and Food Chemistry, Grindelallee 117, D-20146 Hamburg, Germany

² University of Darmstadt, Faculty of Biology, Schnittspahnstrasse 3, D-64287 Darmstadt, Germany

³ University of Potsdam, Department of Food Chemistry, Arthur-Scheunert Allee 114-116, D-14558 Nuthetal, Germany

Keywords

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Summary

In this study we analyzed the isoflavone (IF) content and composition of twelve dietary phytoestrogen supplements commercially available on the German market and compared our results with the manufacturer's label claim. Based on our analysis with HPLC/DAD/MS, we found considerable discrepancies (deviations up to $\pm 50\%$) from the manufacturers claim in the IF content of seven supplements. Four producers do not give any concrete information about the IF content at all and only two products showed a good correspondance between labelling and our measured values. Moreover we investigated the oxidative metabolism of the isoflavone irilone which is one of the three main isoflavones occurring in red clover based dietary supplements by using rat liver microsomes. As a result of this *in vitro* study we could identify five metabolites by HPLC-DAD/MS analysis predominantly containing a pyrogallol moiety.

Introduction

During the past years a growing market has developed for dietary supplements derived from soy and red clover. These preparations, which are mostly addressed to postmenopausal women as a so-called "natural alternative" to hormone replacement therapy, contain IF as bioactive compounds. Apart from their effect of reducing menopausal symptoms IF are associated with various other health benefits such as preventing breast and prostate cancer, lowering the risk of cardiovascular disease or prevention of osteoporosis.

Recent studies, which were performed in the USA, Australia and Finland, revealed that phytoestrogen supplements often contained significantly less IF than the amount claimed on the label [1,2,3].

Our studies focussed on the following three aspects:

- (1) Analytical characterization and quantification of IF in dietary supplements derived from soy and red clover which are available on the German market.
- (2) To evaluate how well the isoflavone content data supplied by the manufactures correspond to our analysis results
- (3) Investigation of the cytochrome P450-catalyzed metabolism of the red clover isoflavone irilone.

Experimentals

(1 and 2) The IF (in the aglycone and in the glucoside form) were extracted from twelve dietary supplements by liquid-liquid extraction. Separation was performed on a Prontosil 120-3-C18-ace-EPS column (Bischoff Chromatography, Leonberg, Germany). Characterization was carried out on an Agilent 1100 HPLC-DAD/MS operating in the ESI positive mode. The quantification was done by HPLC-DAD before and after the enzymatic hydrolysis of the sugar conjugated forms with both, β -glucosidase and β -glucuronidase.

(3) Initial *in vitro* studies on the IF irilone were carried out with rat liver microsomes prepared from male Sprague Dawley rats. Incubations were performed as described by Kulling et al. [4]. In brief, purified irilone (25 μ g) were incubated in a potassium phosphate-buffered solution (total volume of 2 ml) with 3.5 mg microsomal protein in the presence and absence of a NADPH-generating system for 60 minutes at 37°C. The metabolites were extracted with ethyl acetate and were characterized by HPLC-DAD/MS. Daidzein was used as a positive control to check the incubation conditions.

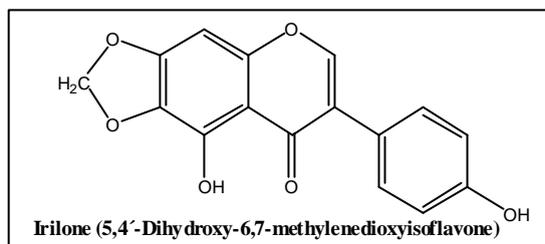
Results

The major isoflavones in the analyzed soy supplements were daidzein and genistein; glycitein was only a minor component. The main IF (> 5% of the total IF amount) of red clover were formononetin (35-55%), biochanin A (20-50%) and irilone (5-10%). Genistein, daidzein, pratensein, pseudobaptigenin, glycitein and prunetin were only found in small amounts less than 5%. A representative HPLC profile of two different isoflavone supplements, made from soy and red clover, is shown in Figure 1.

While soy preparations contain almost no IF aglycones the products based on red clover show an aglycone : glycoside ratio of approximately 5 to 1.

(2) According to our analysis most of the supplements were incorrectly labelled. The deviation between the label claim and the analyzed IF values ranged up to ± 50 %.

(3) The metabolism of the two main red clover IF biochanin A and fomononetin has already been investigated. Major metabolites are the demethylation products genistein and daidzein, respectively [5]. Irilone, the third important IF in red clover, has not been investigated so far. The chemical structure of irilone possesses a methylenedioxy-group and



therefore seems to be a very interesting substance for metabolism studies [6]. Our initial studies show that irilone is a substrate for cytochrome P450 enzymes. The major metabolite was identified as 5,6,7,4'-Tetrahydroxy-IF (M3), which is formed by oxidative demethylation of the methylenedioxy-group. Other metabolites were characterized as 3'-Hydroxyirilone (M5), 8-Hydroxyirilone (M4), 5,6,7,8,4'-Pentahydroxy-IF (M1) and 3',8-Dihydroxyirilone (M2). The relative distribution of the oxidative irilone metabolites is shown in figure 2. Compared to daidzein, which was used as a positive control, irilone was metabolized to a much higher extent by cytochrome P450 enzymes.

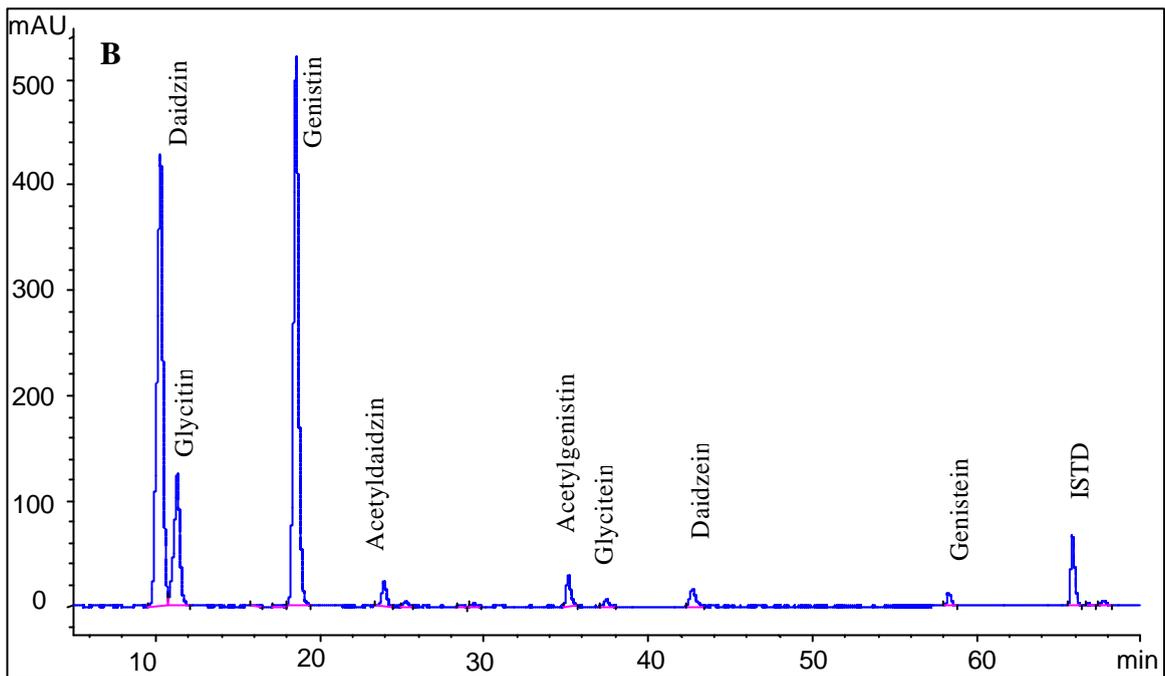
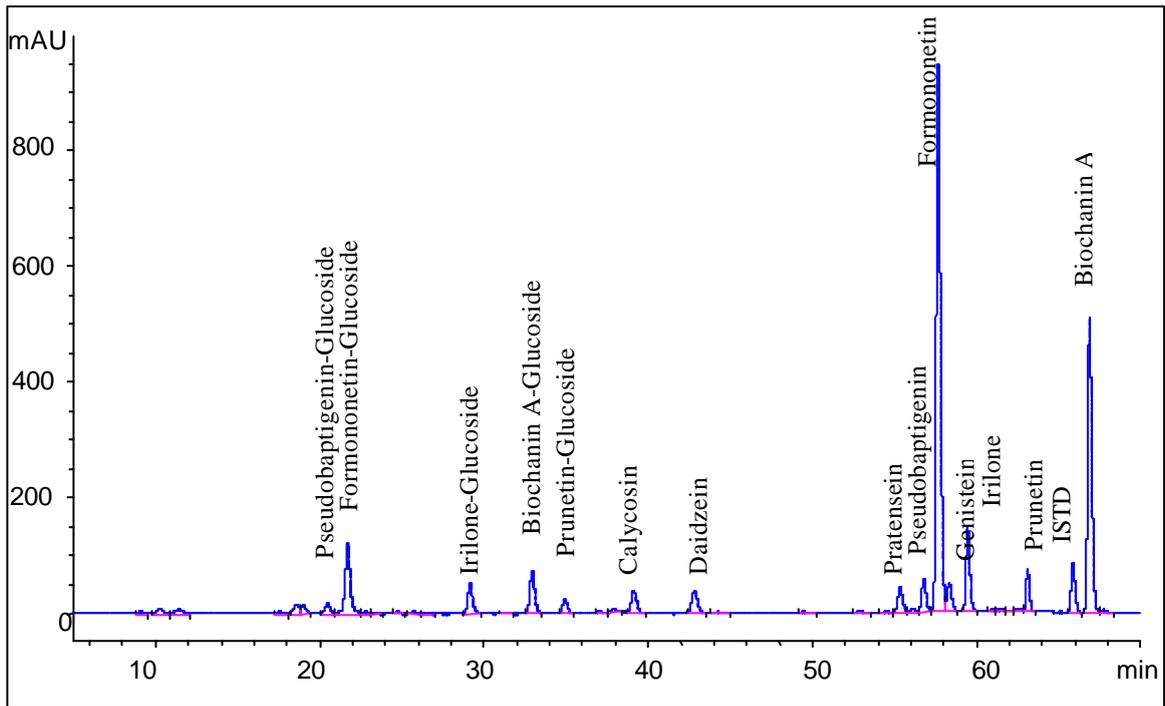


Figure 1. Chromatogram of a typical red clover based (A) and a soy based (B) supplement

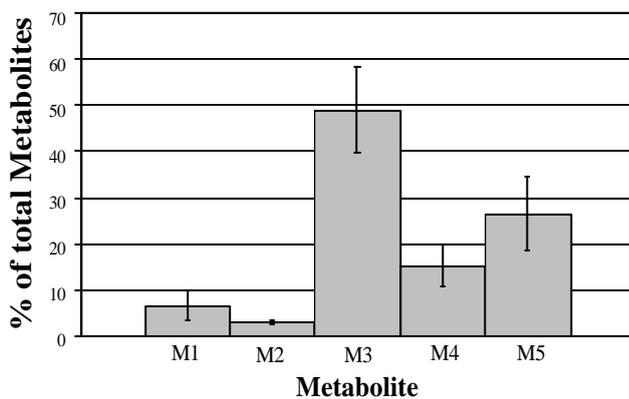


Figure 2. Relative distribution of the five identified irilone metabolites (quantification based)

on absorbance at 260 nm; SD based on trifold analysis).

Discussion

Soy and red clover phytoestrogen supplements show substantial differences in regard to the amount and the composition of bioactive compounds. Main components of soy-based supplements are daidzein and genistein while red clover based products contain the corresponding methoxylated isoflavones formononetin and biochanin A. Another important difference is that soy based supplements mainly contain IF in the glucoside form whereas in the red clover based preparations the aglycones predominate. Such differences in the composition could have a substantial impact on the IF plasma concentration and the IF plasma profile and therefore on the biological effect. Furthermore red clover supplements contain isoflavones like irilone and pseudobaptigenin. Nearly nothing is known about the bioavailability, the metabolism and the biological activity of these compounds. Although they appear in much smaller quantities than the main components this does not mean that they are biologically inactive. Our initial studies show that irilone is an excellent substrate for cytochrome P450 enzymes leading to the formation of oxidative metabolites with a pyrogallol moiety. These metabolites may possess pro-oxidant activities due to their ability to form corresponding quinone structures.

Furthermore, the claimed IF contents and our analyzed values showed large deviations up to $\pm 50\%$ in many cases. Recent studies of isoflavone-containing preparations marketed in the USA and in UK produced similar results to ours with the exception that we found both, supplements with lower and those with a higher IF content than stated on the label.

One reason for the high deviations (in the of the soy preparations with an IF content being labelled too low) could be that the manufactures calculate the IF on the basis of their sugar conjugates and not as the aglycones. In the case of the red clover products it seems questionable which components of the complex mixture (Figure 1A) were used for the calculation of the IF content by the manufactures.

Conclusions

Phytoestrogen supplements of the german market show a large diversity concerning their isoflavone content and their chemical composition although they are labelled with similar advertising statements. Especially red clover preparations contain several isoflavones, which have not been investigated so far.

Since supplements are often used by the consumers for self-medication purpose the danger of overdosing must be taken into account. So far, it could not be excluded that a high intake of phytoestrogen supplements cause adverse health effects - particularly in risk groups like postmenopausal women with precancerous lesions in the mammary gland.

Furthermore consumers expect safe products with a correct labelling, especially in the case of food supplementations mainly employed as "natural" and allegedly more healthy alternatives for a hormonal replacement therapy.

With respect to the consumers safety it therefore seems necessary to assess phytoestrogen dietary supplements like drugs with regard to standardization, quality, effectiveness, potential side effects and product labelling.

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