Absorption of red clover isoflavones in human subjects: results from a pilot study

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In addition to soya-derived preparations, red clover-based dietary supplements have gained considerable interest as an alternative isoflavone (IF) source. While metabolism and bioavailability of the main IF from both sources have already been investigated, studies are still lacking on the biokinetic behaviour of IF, which are present in red clover in minor amounts. In the present pilot study, in which seven volunteers ingested a single dose of a commercial red clover dietary supplement, we focused on the absorption of three such IF, irilone (IRI), prunetin (PRUN) and pseudobaptigenin (PBAP). The compounds were measured as aglycones after enzymatic hydrolysis. A single intake of an amount of as low as 3.8 mg IRI (out of 38.8 mg IF in total) resulted in an IRI plasma concentration of 0.35 (SD 0.16) μM at 6.5 h post-ingestion. Compared to the plasma concentrations found for daidzein (0.39 μM) and genistein (0.06 μM), expected to be the main IF metabolites in plasma, the present findings indicate that IRI might possess a relatively high bioavailability. Furthermore, PRUN and PBAP were detected in human plasma for the first time.

Isoflavones: Irilone: Bioavailability: Red clover: Dietary supplements

Red clover-based extract preparations containing isoflavones (IF) have gained increasing importance especially for postmenopausal women as a target group. Compared to soya with daidzein (DAI), genistein (GEN) and glycitein (GLYC) as the present IF, red clover contains at least seven additional IF, with formononetin (FORM) and biochanin A (BIOA) being the dominating ones. Furthermore, irilone (IRI), prunetin (PRUN), pseudobaptigenin (PBAP), calycosin and pratensein have been detected in red clover. These IF are characterised by the presence and absence of methoxy- and/or methylenedioxy groups attached to the IF skeleton at different positions. The bioavailability and metabolism of soya IF have already been investigated. However, only three studies in human subjects have been published dealing with the biotransformation of the main red clover IF FORM and BIOA with the minor IF being neglected. Howes et al. reported that a daily ingested dose of about 80 mg red clover IF for 2 weeks led to considerable total plasma levels of FORM, BIOA and their demethylated metabolites DAI and GEN, respectively, which occurred in higher concentrations than the parent compounds themselves. This can either be explained by the microbial O-demethylation of FORM and BIOA and a subsequent colonic absorption, or by the oxidative demethylation of FORM and BIOA catalysed by cytochrome P450 enzymes. The aim of the present study was to provide a more comprehensive IF profile in human plasma after intake of a single bolus dose of a commercially available red clover supplement. Special focus was given to the detection of minor IF in order to evaluate their in vivo importance.

Materials and methods

Design of the pilot study

Four male and three female volunteers (24–30 years of age, BMI ranging from 20.2 to 26.5 kg/m²) participated in the study and gave their appropriate consent to the study protocol. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and was approved by the ethics committee of the University of Potsdam. Verbal informed consent was obtained from all subjects and formally recorded. The volunteers were considered to be healthy and had not taken any antibiotics for at least last 6 months. The red clover supplement (MenoStabil, Bad Heilbrunner Naturheilmittel GmbH&Co, Bad Heilbrunn, Germany) used was analysed by LC–UV–ESI(+)–MS following the clean-up procedure as described previously. Two gelatine capsules...
contained approximately 38.8 mg IF in total (calculated as aglycones). The IF content that could be measured based on the available pure standard substances was 8.7 mg BIOA, 18.9 mg FORM, 3.8 mg IRI, 1.3 mg DAI, 1.2 mg GLYC and 0.2 mg GEN.

Volunteers were asked to abstain from any kind of food items or supplements containing IF 3 d before the red clover supplementation. A list of items to be avoided was given to all participants. Two capsules, the daily dosage recommended by the manufacturer, were ingested as a single bolus dose together with a glass of water after fasting overnight and eating an IF-free breakfast. In order to contain costs for the present pilot study and to avoid unnecessary stress for the participants, blood samples were taken by venepuncture from selected volunteers before ingestion and from all volunteers only at a single point in time, i.e. 6.5 h after IF intake. This particular time point was chosen because the peak plasma concentration after oral uptake of the IF aglycones, GEN and DAI, occurs after about 6 h\(^\text{k2,4}\). Thus, for the red clover IF aglycones, the predominant chemical form in the supplement, by electron impact ionisation at 70 eV and an ion source temperature of 200°C. Full scan spectra (mass range 50–650 amu) were recorded. The MS\(^2\) mode was applied using mass-to-charge ratios for precursor ions and daughter ion ranges, respectively: BIOA (413; 206–413), DAI (398; 199–398), FORM (340; 170–340), IRI (471; 235–471) and IRI (427; 213–427). Quantifications were carried out using GLYC as an internal standard compound as well as external calibration curves for FORM, BIOA, IRI, DAI and GEN by spiking IF-free control plasma with the respective IF. The minor red clover IF PRUN and PBAP were investigated as to their presence in the plasma samples, but were not quantified due to the lack of standard compounds with sufficient purity.

### Chemicals

The chemicals used were of the highest grade available. GLYC, DAI and GEN were purchased from LC Laboratories (Woburn, MA, USA), IRI was purchased from LGC...
Promochem (Wesel, Germany), and PBAP was purchased from APIN Chemicals (Abingdon, UK). BIOA, FORM, PRUN and dimethylsulphoxide as well as β-glucuronidase (from Helix Pomatia) were obtained from Sigma-Aldrich Chemical Co. (Deisenhofen, Germany). Bis(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane 99:1 (v/v) was from Macherey-Nagel (Dueren, Germany). All IF standard compounds except PBAP and PRUN had a purity above 98% according to LC–UV–ESI(+)-MS analysis.

## Results and discussion

### Distribution pattern of isoflavones in the red clover supplement

The analysis of the hydrolysed red clover supplement showed at least nine different IF with two dominating peaks representing the main red clover IF, FORM and BIOA, as well as three smaller peaks for DAI, GEN and IRI in the MS/MS chromatogram (Fig. 1(a)). PRUN and PBAP led to less intense peaks as shown in Fig. 1(b).

### Distribution pattern of isoflavones in human plasma

The plasma IF profiles, measured 6.5 h after intake of the two capsules containing 38.8 mg IF in total, were similar for all seven subjects. However, the plasma profiles were clearly different from the IF pattern observed in the red clover supplement. As expected, the two main red clover IF, FORM and BIOA, were demethylated to a great extent to form DAI and GEN, respectively. A representative chromatogram is shown in Fig. 1(a). The mean plasma concentrations for the male and female subjects are given in Table 1. The plasma levels, their body weight and the mean plasma levels per kilogram body weight are given in Table 1. Howes et al. (6), our data point out that the IF composition of a dietary supplement has a major influence on the resulting IF pattern in the plasma. Howes et al. used a red clover supplement with a low FORM content (FORM:BIOA ratio of 0.65). The daily IF intake of about 80 mg led to maximum DAI and GEN plasma levels of 0.25 and 0.42 μM, respectively. Correspondingly, the present study with a single bolus intake of about 40 mg IF high in FORM (FORM:BIOA ratio of 2:17) led to plasma concentrations of the demethylated metabolites DAI and GEN of 0.39 and 0.06 μM, respectively.

### Table 1. Detected isoflavone (IF) plasma levels in each of the seven subjects at 6.5 h post-intake of a single dosage of a red clover supplement (the detailed composition is given in Materials and methods) and as mean values for the female, male and entire group of participants

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Body weight (kg)</th>
<th>FORM</th>
<th>BIOA</th>
<th>DAI</th>
<th>GEN</th>
<th>IRI</th>
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<tbody>
<tr>
<td>Female 1</td>
<td>70</td>
<td>144</td>
<td>21</td>
<td>347</td>
<td>79</td>
<td>197</td>
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<td>Female 2</td>
<td>72</td>
<td>68</td>
<td>10</td>
<td>385</td>
<td>52</td>
<td>250</td>
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<tr>
<td>Female 3</td>
<td>64</td>
<td>34</td>
<td>9</td>
<td>158</td>
<td>36</td>
<td>179</td>
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<td>70</td>
<td>187</td>
<td>10</td>
<td>493</td>
<td>58</td>
<td>589</td>
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<tr>
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<td>140</td>
<td>26</td>
<td>592</td>
<td>94</td>
<td>394</td>
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<td>56</td>
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<tr>
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<td>132</td>
<td>44</td>
<td>15</td>
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<tr>
<td>Group</td>
<td>111</td>
<td>52</td>
<td>14</td>
<td>385</td>
<td>63</td>
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</table>

Interestingly, we observed higher IF plasma levels for the male volunteers, especially in the case of IRI (Table 1). However, the number of volunteers is too small to even speculate that bioavailability is higher in men.

Fig. 1(b) illustrates different absorption rates of the structural isomers BIOA and PRUN (4',5,7-trihydroxy-IF either being methylated in position C4' or C7, respectively) by showing the relative intensity of both isomers in the red clover extract as well as in a representative plasma sample, whereas BIOA is clearly more abundant in the red clover extract, and PRUN dominates in relation to BIOA in the plasma samples. Similarly for FORM, DAI and IRI (Fig. 1(a)), clear differences in the plasma pattern compared to the red clover extract pattern were also observed. These data demonstrate that the plasma concentration of a single IF cannot be assessed based on its concentration in the respective dietary supplement. Further detailed investigations on the bioconversion of all constituents have to be carried out. The need for more thorough investigations is also emphasised by the detection of PBAP in all plasma samples indicating that also minor IF are systemically available after intake of red clover dietary supplements.

The plasma levels of IRI were unexpectedly high. This observation might be explained by the results from a recent study in which we investigated the degradability of IRI compared to GEN by human faecal microbiota. We were able to show that in contrast to GEN, IRI is almost resistant to such conversion (11). Therefore, it stands to reason that a relatively high amount of the parent compound IRI is available for absorption in the intestine, especially in the colon.

Along with the results of the long-term study by Howes et al., our data point out that the IF composition of a dietary supplement has a major influence on the resulting IF pattern in the plasma. Howes et al. used a red clover supplement with a low FORM content (FORM:BIOA ratio of 0.65). The daily IF intake of about 80 mg led to maximum DAI and GEN plasma levels of 0.25 and 0.42 μM, respectively. Correspondingly, the present study with a single bolus intake of about 40 mg IF high in FORM (FORM:BIOA ratio of 2:17) led to plasma concentrations of the demethylated metabolites DAI and GEN of 0.39 and 0.06 μM, respectively.

**Formononetin; BIOA, biochanin A; DAI, daidzein; GEN, genistein; IRI, irilone.**
Compared to the present results, Setchell et al. (4) reported slightly lower plasma levels for FORM, BIOA, DAI and GEN after a single bolus intake of a preparation (about 40 mg total IF) also low in FORM. However, that analysis was performed for a single subject only.

Conclusion
The present study shows for the very first time that the IF IRI is bioavailable to a high extent. IRI concentrations in commercially available red clover supplements are sufficient to lead to physiologically relevant plasma concentrations (12). Along with our recent findings, we propose that the methylenedioxy bridge attached to the A ring of the IF skeleton acts as a protective group against degradation of IRI by the human microbiota. Furthermore, PRUN and PBAP could be detected in all subjects’ blood plasma. So far, most of these minor IF constituents have not been toxicologically investigated. IRI and PBAP bear a methylenedioxyphenyl group, which is a structural characteristic known to exert biologically relevant effects, such as inducing hepatic cytochrome P450 enzyme expression (13). Thus, further studies on the bioavailability and the metabolism of the red clover IF are advised.

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References