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Preparative isolation of isoflavones from soy and red clover

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Numerous studies have demonstrated the potential of isoflavones occurring in soy (*Glycine max* L.) and red clover (*Trifolium pratense* L.) to alleviate climacteric complaints. They have also shown beneficial effects on the cardiovascular system and in the prevention of osteoporosis. As a result, several companies offer nutraceuticals based on soy or red clover extracts. The aim of the present study was the isolation of pure isoflavones on a preparative scale, in order to obtain standards for biological tests and for the quantification of isoflavones in nutraceuticals and foods. High-speed countercurrent chromatography, a special type of liquid–liquid partition chromatography, was applied to the preparative isolation of isoflavones. By using this technique the major monoglucosylated and acetylated isoflavones from soy extracts were obtained after a cleaning-up step on Amberlite XAD-7 material. Furthermore, it was possible to isolate isoflavone aglycones as well as glycosides from a red clover extract. Purity and identity of the isolated isoflavones were confirmed by HPLC with DAD, HPLC-ESI multiple-MS, and NMR spectroscopy.

Keywords: Countercurrent Chromatography / Isoflavones / Red clover / Soy

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1 Introduction

Isoflavones are widely distributed in the plant family of Leguminosae, in particular in soy (*Glycine max* L.) and red clover (*Trifolium pratense* L.). Soybeans and soy-derived foods are important for the human nutrition and contain different conjugated forms (β-glucoside, acetylglucoside, and malonylglucoside) of the isoflavone aglycones genistein, daidzein, and glycitein [1, 2]. Red clover is mainly a forage plant for cattle and sheep, but was as well used as a medicinal herb by the indigenous people of North America. This plant contains a wide variety of isoflavones structurally different from the ones encountered in soy (*e.g.* formononetin and biochanin A) [3].

Based on their structural similarity to the mammalian estrogen 17β-estradiol and the resulting weak estrogenic activities, isoflavones are an important group of phytoestrogens [4]. Phytoestrogens are considered to have beneficial effects in the prevention of cancer, cardiovascular diseases, and osteoporosis [5]. Furthermore, the potential of isoflavones occurring in soy and red clover to alleviate climacteric complaints, particularly hot flushes and tachycardic attacks has been demonstrated in several studies [6, 7]. Due to the possible benefits of phytoestrogens for the human health, several companies offer nutraceuticals based on soy and red clover extracts. The concentration of isoflavones in these products is highly variable [8]. Moreover, recent studies revealed potential adverse effects of high phytoestrogen intake due to possible genotoxicity (and cytotoxicity) of, *e.g.* genistein [9]. Therefore, it is important to obtain pure standards of isoflavones to be able to accomplish studies about the bioavailability, bioactivity, and metabolism of these compounds. Furthermore, the standards will enable the development of a selective analytical method for the quantification of isoflavones in nutraceuticals and various foods. By this approach, identification of the constituents of commercially available dietary supplements and comparison of their actual content of isoflavones with the declaration on the label will be feasible.

HPLC is commonly used for the analyses of isoflavones. However, due to the low solubility of some isoflavones, this method is not suitable for the isolation of isoflavones on a preparative scale. Instead, we were using high-speed countercurrent chromatography (HSCCC), which is a support-free technique based on liquid–liquid partition chromato-

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Abbreviations: CCC, countercurrent chromatography; **HSCCC,** high-speed countercurrent chromatography; **MTBE,** methyl-*tert*-butylether

graphy. HSCCC is the ideal method for the nondestructive isolation and enrichment of labile natural compounds [10]. In HSCCC, a liquid stationary phase is loaded into a teflon tubing that is wrapped in multiple layers around a coil. Concomitantly with the injection of the sample, a second immiscible phase (mobile phase) is pumped through the tubing and rotation of the coil at 600–1000 rpm in a planetary motion is started. Due to the resulting force fields, alternating mixing and demixing of the two immiscible phases occur and the analytes are efficiently distributed according to their partition coefficients [11]. The chromatographic separation was monitored by UV-detection. Some fractions were further purified by semipreparative HPLC and the isolated isoflavones were identified by ESI-MS and NMR analyses.

2 Materials and methods

2.1 Chemicals

ACN was of HPLC purity; hexane, ethyl acetate, and *n*-butanol were of analytical grade. Methanol and methyl-*tert*-butylether (MTBE) were redistilled prior to use. Formic acid was obtained from Roth (Karlsruhe, Germany), perdeuterated dimethylsulfoxide from Aldrich (Milwaukee, USA).

2.2 Plant material and extraction

Toasted soy flour was purchased from a local supplier, and a raw extract (ethanol/water) of red clover was obtained from Pfannenschmidt (Hamburg, Germany). Toasted soy flour was defatted twice with hexane, and the dried flour was then extracted with 75% aqueous methanol. After evaporation of the solvent under vacuo, the raw extract was freeze-dried.

2.3 SPE

The extracts were cleaned and enriched by SPE using Amberlite XAD-7 material (Fluka, Buchs, Switzerland) that was filled in a glass column (45 cm × 5 cm). After applying the filtered aqueous crude extract, the resin was washed with water to remove proteins, sugars, organic acids, and salts. Phenolic compounds like isoflavones were retained by the resin and subsequently eluted with methanol. The eluate was concentrated in vacuo, diluted with water, and freeze-dried to give an isoflavone-enriched XAD-7 extract, which was used for the HSCCC separation.

2.4 Countercurrent chromatography (CCC) instrumentation

The HSCCC was manufactured by Pharma-Tech Research (Baltimore, Maryland, USA) and equipped with three preparative coils, connected in series (total volume approximately 850 mL, id 2.6 mm). Depending on the application, the revolution speed varied between 600 and 800 rpm, and flow rates from 2.5 to 3.2 mL/min (pumped with Biotronik HPLC pump BT 3020). Detection was carried out at $\lambda = 260$, 254, or 315 nm (Knauer Variable Wavelength Monitor). Due to the low solubility of isoflavones, a small amount (1–2 mL) of DMSO was first applied to the samples and then for a complete dissolution a 1:2 mixture of light and heavy phases of the respective CCC solvent system. After membrane filtration the sample solution was injected *via* a sample loop. The separations were performed in the head-to-tail elution mode with the organic layer acting as stationary phase.

2.5 Analytical HPLC with DAD

For HPLC analyses a PU-980 Intelligent HPLC pump equipped with a DG-980-50-3-line degasser, a LG-980-02 ternary gradient unit, and a MD-1510 multiwavelength detector was used (Jasco, Gross-Umstadt, Germany). Samples were injected *via* a Rheodyne 7125 injection valve, equipped with a 20 µL sample loop. Separations were carried out on a Luna C18(2) column (Phenomenex®, Aschaffenburg, Germany, 5 µm, 250 mm × 4.6 mm with guard column). Solvents were 0.1% formic acid (A) and ACN (B), the flow rate was 0.8 mL/min. The binary gradient for soy isoflavones was % A: 0 min, 85%; 30 min, 76%; 40 min, 10%; 45 min, 85%. For analyses of isoflavones of red clover the gradient was modified into % A: 0 min, 85%; 25 min, 76%; 40 min, 55%; 50 min, 59%; 65 min, 10%; 70 min, 85%. Data about the purity of isoflavone fractions were based on the DAD chromatogram using $\lambda = 260$ nm as detection wavelength.

2.6 Preparative HPLC

Separations were performed isocratically on a Luna C18(2) column (Phenomenex, 5 µm, 250 mm × 10.0 mm with guard column). Solvents were a binary mixture of 0.1% formic acid and ACN, and the flow rate was set at 6.0 mL/min. The appropriate HSCCC fractions were diluted in DMSO and the above-mentioned binary solvent mixture (1:2 v/v). The separated isoflavones were collected and lyophilized.

2.7 HPLC with ESI-MS

HPLC-MS analyses of CCC fractions and purified isoflavones were performed on a Bruker Esquire LC-MS system (Bruker Daltonik, Germany). The HPLC system consisted of a System 1100 Binary Pump G1312A (Agilent, Böblingen, Germany), a Rheodyne 7725i injection valve with 20 µL loop (Rheodyne, Rohnert Park, California, USA), and a Lichrograph L-4000 UV/Vis detector (Merck Hitachi, Tokyo, Japan), the UV-chromatograms were recorded with a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan). The LC part was controlled *via* ChemStation software version A.06.01; MS data were processed by Esquire NT 4.0 software. The MS parameters were set as follows: positive ionization mode; capillary –1500 V, end plate offset –500 V; capillary exit offset 70 V; skimmer 1: 25 V, skimmer 2: 10 V; drying gas N_2, drying temperature 310°C, gas flow 9 L/min; nebulizer 40 psi.

2.8 NMR spectroscopy

All ^{1}H- and ^{13}C-NMR experiments were performed on a Bruker AMX 300 spectrometer (Bruker Biospin, Rheinstetten, Germany) at 300.0 and 75.5 MHz, respectively. The spectra were recorded in perdeuterated dimethylsulfoxide and calibrated to the solvent signals (δ ^{1}H: 2.49 (ppm); ^{13}C: 39.7 (ppm)). Data were processed by WIN-NMR software version 6.1.0.0.

2.9 Structural identification of the isolated isoflavones

2.9.1 Daidzin

ESI-MS (positive mode): *m/z* 417 $[M + H]^+$, MS^2 *m/z* 255 [M + H-anhydroglucose]$^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.12–3.31 (3H, m, H2″/H3‴/H4″); 3.42–3.47 (2H, m, H5″/H6$_a$″); 3.72 (1H, m, H6$_b$″); 4.54 (1H, m, sugar-O<u>H</u>); 4.99 (1H, d, *J* = 5.0 Hz, sugar-O<u>H</u>); 5.05 (1H, s_{br}, sugar-O<u>H</u>); 5.08 (1H, d, *J* = 7.0 Hz, H1″); 5.35 (1H, d, *J* = 3.5 Hz, sugar-O<u>H</u>); 6.81 (2H, m, AA′-BB′, H3′/H5′); 7.14 (1H, dd, J_1 = 9.0 Hz, J_2 = 2.0 Hz, H6); 7.22 (1H, d, *J* = 2.0 Hz, H8); 7.40 (2H, m, AA′-BB′, H2′/H6′); 8.04 (1H, d, *J* = 9.0 Hz, H5); 8.36 (1H, s, H2); 9.47 (1H, s, 4′-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 60.6 (C6″); 69.6 (C4″); 73.0 (C2″); 76.4 (C3″); 77.1 (C5″); 100.0 (C1″); 103.4 (C8); 114.9 (C3′/C5′); 115.5 (C6); 118.4 (C10); 122.2 (C1′); 123.6 (C3); 126.8 (C5); 129.9 (C2′/C6′); 153.1 (C2); 156.9 (C4′); 157.2 (C9); 161.3 (C7); 174.6 (C4).

2.9.2 Glycitin

ESI-MS (positive mode): *m/z* 447 $[M + H]^+$, MS^2 *m/z* 285 [M + H-anhydroglucose]$^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.15–3.31 (3H, m, H2″/H3″/H4″); 3.43–3.49 (2H, m, H5″/H6$_a$″); 3.71 (1H, m, Hz, H6$_b$″); 3.88 (3H, s, 6-O<u>CH</u>$_3$); 4.53 (1H, m, sugar-O<u>H</u>); 4.99 (1H, d, *J* = 5.0 Hz, sugar-O<u>H</u>); 5.05 (1H, s_{br}, sugar-O<u>H</u>); 5.16 (1H, d, *J* = 8.0 Hz, H1″); 5.30 (1H, d, *J* = 3.0 Hz, sugar-O<u>H</u>); 6.81 (2H, m, AA′-BB′, H3′/H5′); 7.31 (1H, s, H8); 7.40 (2H, m, AA′-BB′, H2′/H6′); 7.48 (1H, s, H5); 8.35 (1H, s, H2); 9.46 (1H, s, 4′-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 55.8 (O<u>CH</u>$_3$); 60.6 (C6″); 69.6 (C4″); 73.0 (C2″); 76.7 (C3″); 77.2 (C5″); 99.7 (C1″); 103.4 (C8); 104.8 (C5); 114.9 (C3′/C5′); 117.8 (C10); 122.5 (C1′); 123.1 (C3); 129.9 (C2′/C6′); 147.4 (C6); 151.1 (C9); 151.5 (C7); 152.9 (C2); 157.1 (C4′); 174.3 (C4).

2.9.3 Genistin

ESI-MS (positive mode): *m/z* 433 $[M + H]^+$, MS^2 m/z 271 [M + H-anhydroglucose]$^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.13–3.22 (1H, m, H4″), 3.44 (2H, m, H2″/H3″); 3.69 (2H, d, *J* = 5.5 Hz, H5″/H6$_a$″); 3.72 (1H, d, *J* = 5.0 Hz, H6$_b$″); 4.54 (1H, m, sugar-O<u>H</u>); 4.99 (1H, d, *J* = 6.0 Hz, H1″); 5.04 (1H, m, sugar-O<u>H</u>); 5.06 (1H, m, sugar-O<u>H</u>); 5.33 (1H, d, *J* = 2.0 Hz, sugar-O<u>H</u>); 6.46 (1H, d, *J* = 2.5 Hz, H6); 6.71 (1H, d, *J* = 2.5 Hz, H8); 6.82 (2H, m, AA′-BB′, H3′/H5′); 7.39 (2H, m, AA′-BB′, H2′/H6′); 8.40 (1H, s, H2); 9.55 (1H, s, 4′-O<u>H</u>); 12.90 (1H, s_{br}, 5-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) 60.7 (C6″); 69.6 (C4″); 73.0 (C2″); 76.4 (C3″); 77.2 (C5″); 94.6 (C8); 99.6 (C6); 99.9 (C1″); 106.0 (C10); 114.8, 115.2 (C3′/C5′); 120.9 (C3); 122.5 (C1′); 129.9, 130.2 (C2′/C6′); 154.7 (C2); 157.2 (C9); 157.4 (C4′); 161.6 (C5); 163.0 (C7); 180.4 (C4).

2.9.4 6″-O-Acetyl-daidzin

ESI-MS (positive mode): *m/z* 459 $[M + H]^+$, MS^2 *m/z* 255 [M + H-anhydroacetylglucose]$^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 2.01 (3H, s, C<u>H</u>$_3$-CO); 3.15–3.38 (3H, m, H2″/H3″/H4″); 3.71–3.77 (1H, m, H5″); 4.07 (1H, m, H6$_a$″); 4.34 (1H, m, H6$_b$″); 5.14 (1H, d, *J* = 7.5 Hz, H1″); 5.18 (1H, d, *J* = 7.5 Hz, sugar-O<u>H</u>); 5.28 (1H, d, *J* = 5.0 Hz, sugar-O<u>H</u>); 5.45 (1H, d, *J* = 4.0 Hz, sugar-O<u>H</u>); 6.84 (2H, m, AA′-BB′, H3′/H5′); 7.13 (1H, dd, J_1 = 9.0 Hz, J_2 = 2.0 Hz, H6); 7.21 (1H, d, *J* = 2.0 Hz, H8); 7.40 (2H, m, AA′-BB′, H2′/H6′); 8.05 (1H, d, *J* = 9.0 Hz, H5); 8.36 (1H, s, H2); 9.48 (1H, s, 4′-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 20.5 (<u>CH</u>$_3$-CO); 63.2 (C6″); 69.7 (C4″); 73.0 (C2″); 73.7 (C3″); 76.2 (C5″); 99.7 (C1″); 103.4 (C8); 114.9 (C3′/C5′); 115.4 (C6); 118.5 (C10); 122.2 (C1′); 123.7 (C3); 126.9 (C5); 129.9 (C2′/C6′); 153.2 (C2); 156.9 (C4′); 157.2 (C9); 161.1 (C7); 170.1 (CH$_3$-<u>C</u>O); 174.6 (C4).

2.9.5 6″-O-Acetyl-genistin

ESI-MS (positive mode): *m/z* 475 $[M + H]^+$, MS^2 *m/z* 271 [M + H-anhydroacetylglucose]$^+$. ^{1}H-NMR (300 MHz,

DMSO-d_6,): δ (ppm) = 2.01 (3H, s, $C\underline{H}_3$-CO); 3.13–3.32 (3H, m, H2″/H3″/H4″); 3.69–3.76 (1H, m, H5″); 4.06 (1H, m, $H6_a$″); 4.31 (1H, m, $H6_b$″); 5.10 (1H, d, J = 7.0 Hz, H1″); 5.17 (1H, d, J = 3.5 Hz, sugar-O<u>H</u>); 5.27 (1H, d, J = 5.5 Hz, sugar-O<u>H</u>); 5.42 (1H, d, J = 5.0 Hz, sugar-O<u>H</u>); 6.47 (1H, d, J = 2.0 Hz, H6); 6.69 (1H, d, J = 2.0 Hz, H8); 6.82 (2H, m, AA′-BB′, H3′/H5′); 7.39 (2H, m, AA′-BB′, H2′/H6′); 8.40 (1H, s, H2); 9.55 (1H, s, 4′-O<u>H</u>); 12.90 (1H, s_{br}, 5-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 20.5 ($\underline{C}H_3$-CO); 63.2 (C6″); 69.8 (C4″); 72.9 (C2″); 73.7 (C3″); 76.1 (C5″); 94.5 (C8); 99.4 (C6); 99.5 (C1″); 106.1 (C10); 115.0 (C3′/C5′); 120.9 (C3); 122.5 (C1′); 130.0 (C2′/C6′); 154.5 (C2); 157.1 (C9); 157.4 (C4′); 161.6 (C5); 162.7 (C7); 170.1 (CH_3-$\underline{C}$O); 180.4 (C4).

2.9.6 Formononetin

ESI-MS (positive mode): *m/z* 269 $[M + H]^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.78 (3H, s, 4′-O$\underline{C}H_3$); 6.85 (1H, d, J = 2.0 Hz, H8); 6.92 (1H, dd, J_1 = 9.0 Hz, J_2 = 2.0 Hz, H6); 6.98 (2H, m, AA′-BB′, H3′/H5′); 7.49 (2H, m, AA′-BB′, H2′/H6′); 7.96 (1H, d, J = 9.0 Hz, H5); 8.31 (1H, s, H2); 10.8 (1H, s, 7′-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 55.1 (O$\underline{C}H_3$); 102.0 (C8); 113.5 (C3′/C5′); 115.1 (C6); 116.4 (C10); 123.1 (C1′); 124.2 (C3); 127.1 (C5); 129.9 (C2′/C6′); 152.9 (C2); 157.4 (C7); 158.9 (C9); 162.6 (C4′); 174.5 (C4).

2.9.7 Irilone

ESI-MS (positive mode): *m/z* 299 $[M + H]^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 6.17 (2H, s, O$\underline{C}H_2$O); 6.82 (2H, m, AA′-BB′, H3′/H5′); 6.85 (1H, s, H8); 7.38 (2H, m, AA′-BB′, H2′/H6′); 8.40 (1H, s, H2); 9.53 (1H, s, 4′-O<u>H</u>); 12.88 (1H, s, 5–O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 89.4 (C8); 102.7 (O$\underline{C}H_2$O); 107.4 (C10); 115.0 (C3′/C5′); 120.8 (C3); 122.1 (C1′); 130.1 (C2′/C6′); 141.3 (C6); 152.9 (C9); 153.9 (C5); 154.3 (C2); 157.4 (C7/C4′); 180.8 (C4).

2.9.8 Prunetin

ESI-MS (positive mode): *m/z* 285 $[M + H]^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.86 (3H, s, 7′-O$\underline{C}H_3$); 6.40 (1H, d, J = 2.0 Hz, H6); 6.64 (1H, d, J = 2.0 Hz, H8); 6.82 (2H, m, AA′-BB′, H3′/H5′); 7.38 (2H, AA′-BB′, H2′/H6′); 8.38 (1H, s, H2); 9.58 (1H, s, 4′-O<u>H</u>); 12.93 (1H, s, 5-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 56.0 (O$\underline{C}H_3$); 92.3 (C8); 97.9 (C6); 105.4 (C10); 115.0 (C3′/C5′); 120.9 (C1′); 122.5 (C3); 130.0 (C2′/C6′); 154.2 (C2); 154.2 (C9); 157.4 (C4′); 161.7 (C7); 165.1 (C5); 180.3 (C4).

2.9.9 Biochanin A

ESI-MS (positive mode): *m/z* 285 $[M + H]^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.78 (3H, s, 4′-O$\underline{C}H_3$); 6.22 (1H, d, J = 2.0 Hz, H6); 6.39 (1H, d, J = 2.0 Hz, H8); 6.99 (1H, m, AA′-BB′, H3′/H5′); 7.49 (2H, m, AA′-BB′, H2′/H6′); 8.35 (1H, s, H2); 12.89 (1H, s, 5-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 55.1 (O$\underline{C}H_3$); 93.6 (C8); 98.9 (C6); 104.8 (C10); 113.6 (C3′/C5′); 121.9 (C1′); 122.8 (C3); 130.0 (C2′/C6′); 154.1 (C2); 157.5 (C7); 159.1 (C9); 161.9 (C5); 164.2 (C4′); 180.0 (C4).

2.9.10 Ononin

ESI-MS (positive mode): *m/z* 431 $[M + H]^+$, MS^2 m/z 269 [M + H-anhydroglucose]$^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.12–3.31 (3H, m, H2″/H3″/H4″); 3.42–3.47 (2H, m, H5″/$H6_a$″); 3.72 (1H, m, $H6_b$″); 3.79 (1H, s, 4′-O$\underline{C}H_3$); 4.56 (1H s_{br}, sugar-O<u>H</u>); 5.09 (3H, d, J = 11.5 Hz, H1″/sugar-O<u>H</u>/sugar-O<u>H</u>); 5.39 (1H, s_{br}, sugar-O<u>H</u>); 6.99 (2H, m, AA′-BB′, H3′/H5′); 7.14 (1H, dd, J_1 = 9.0 Hz, J_2 = 2.0 Hz, H6); 7.23 (1H, d, J = 2.0 Hz, H8); 7.53 (2H, m, AA′-BB′, H2′/H6′); 8.05 (1H, d, J = 9.0 Hz, H5); 8.41 (1H, s, H2). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 55.06 (O$\underline{C}H_3$); 60.6 (C6″); 69.6 (C4″); 73.1 (C2″); 76.4 (C3″); 77.2 (C5″); 100.0 (C1″); 103.4 (C8); 113.5 (C3′/C5′); 115.5 (C6); 118.4 (C10); 123.3 (C1′); 123.9 (C3); 126.8 (C5); 129.9 (C2′/C6′); 153.5 (C2); 156.9 (C9); 159.0 (C4′); 161.4 (C7); 174.5 (C4).

2.9.11 Sissotrin

ESI-MS (positive mode): *m/z* 447 $[M + H]^+$, MS^2 m/z 285 [M + H-anhydroglucose]$^+$. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.13–3.24 (1H, m, H4″), 3.43 (2H, m, H2″/H3″); 3.49 (1H, m, $H6_b$″); 3.69 (1H, m, H5″); 3.72 (1H, m, $H6_a$″); 3.79 (3H, s, 7-O$\underline{C}H_3$); 4.53 (1H, m, sugar-O<u>H</u>); 4.99 (1H, d, J = 5.0 Hz, H1″); 5.04 (1H, d, $J \approx$ 1.0 Hz, sugar-O<u>H</u>); 5.06 (1H, d, J = 6.0 Hz, sugar-O<u>H</u>); 5.33 (1H, d, J = 4.0 Hz, sugar-O<u>H</u>); 6.46 (1H, d, J = 2.0 Hz, H6); 6.72 (1H, d, J = 2.0 Hz, H8); 7.00 (2H, m, AA′-BB′, H3′/H5′); 7.52 (2H, m, AA′-BB′, H2′/H6′); 8.45 (1H, s, H2); 12.81 (1H, s_{br}, 5-O<u>H</u>). ^{13}C-NMR (75.5 MHz, DMSO-d_6): δ (ppm) = 55.1 (O$\underline{C}H_3$); 60.6 (C6″); 69.6 (C4″); 73.0 (C2″); 76.4 (C3″); 77.1 (C5″); 94.5 (C8); 99.6 (C6); 99.9 (C1″); 106.0 (C10); 113.0 (C3′/C5′); 122.2 (C1′); 122.6 (C3); 130.1 (C2′/C6′); 154.7 (C2); 157.1 (C10); 159.2 (C4′); 161.6 (C5); 163.0 (C7); 180.3 (C4).

3 Results

3.1 Isolation of isoflavones from soy flour

For the isolation of isoflavones from soy, the XAD-7 extract was subjected to HSCCC separation. First the solvent sys-

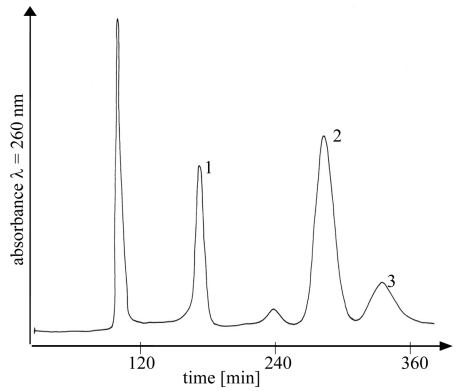

Figure 1. HSCCC separation of isoflavones from soy. See text for details.

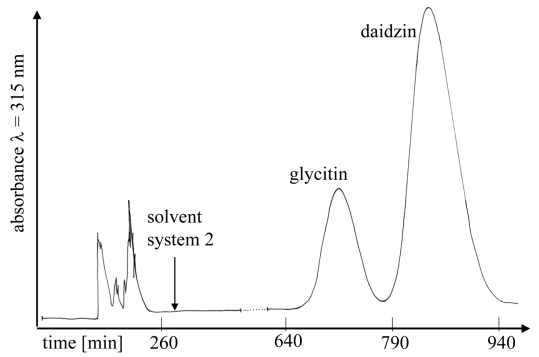

Figure 2. HSCCC separation of the glycitin/daidzin mixture of fraction 1. See text for details.

tem MTBE/ACN/water (2/2/3 v/v/v) at a flow rate of 3.2 mL/min and a rotation speed of 600 rpm was applied. Detection was carried out at $\lambda = 260$ nm. Three major fractions were obtained (Fig. 1). Whereas fractions 2 and 3 contained genistin and 6″-*O*-acetyldaidzin in pure form [12], fraction 1 was still a mixture of daidzin and glycitin.

In addition, 6″-*O*-acetylgenistin was obtained from the coil residue. In order to further separate the fraction 1, the freeze-dried sample was subjected to semipreparative HPLC, using the solvent system 0.1% aqueous formic acid/ACN (38/62 v/v).

In this way, daidzin could be partly enriched. In order to achieve a complete separation of both compounds, a second HSCCC separation, using a "one-step gradient solvent system" was performed. The initial biphasic solvent system was MTBE/*n*-butanol/ACN/water (2/1/2/5 v/v/v/v). After 280 min, the mobile phase (the more dense layer) was replaced with a mixture of MTBE-saturated water and ACN (10/1 v/v) at a flow rate of 2.5 mL/min. For detection the wavelength $\lambda = 315$ nm was applied to monitor the separation of the two compounds (Fig. 2).

In this way, both isoflavones (daidzin and glycitin) could be obtained in pure form. Separation of 100 mg of the mixture of daidzin and glycitin (HSCCC from soy flour, fraction 1) yielded 86 mg of daidzin and 6 mg of glycitin, respectively.

3.2 Isolation of isoflavones from red clover

The HSCCC separation of a lyophilized XAD-7 extract of red clover is depicted in Fig. 3. The application of the solvent system hexane/ethyl acetate/methanol/water (6/5/6/5 v/v/v/v) at a flow rate of 3.0 mL/min and a rotational speed of 800 rpm resulted in the isolation of the major aglycone isoflavones of red clover (Fig. 3). Biochanin A was obtained with a purity of 96% and formononetin with a purity of 82%. Subsequent semipreparative HPLC of the other fractions resulted in an isolation of pure prunetin and irilone.

HSCCC separation of isoflavone glycosides from red clover was carried out with the two-phase solvent system MTBE/ACN/water (6/3/8 v/v/v). The rotational speed of this separation was 600 rpm and the flow rate of the mobile phase 2.8 mL/min (Fig. 4). Six fractions were obtained. Fraction 3 had to be further purified by semipreparative HPLC and contained 85% of ononin. Fraction 6 yielded the isoflavone glucoside sissotrin in a purity of 97%.

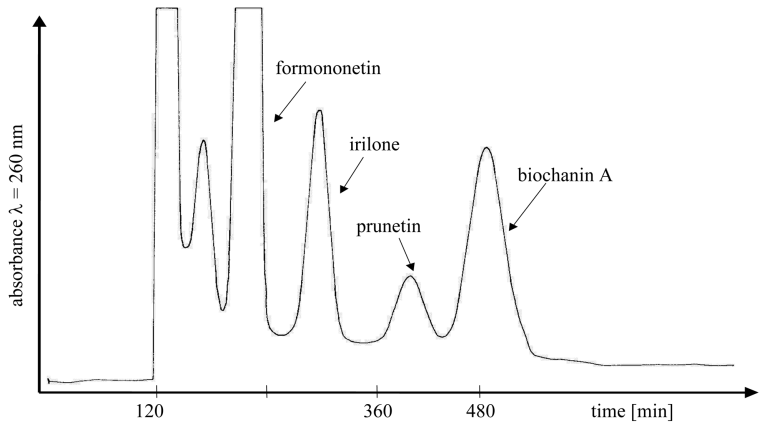

Figure 3. HSCCC separation of the major isoflavone aglycones from red clover. See text for details.

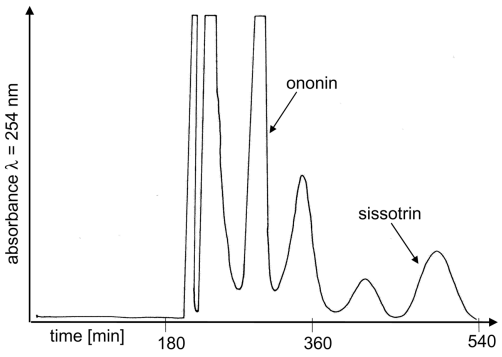

Figure 4. HSCCC separation of isoflavone glycosides from red clover. See text for details.

compound	R_1	R_2	R_3	R_4
daidzin	H	H	H	glc
glycitin	H	H	OMe	glc
genistin	H	OH	H	glc
6′′-*O*-acetyl-daidzin	H	H	H	ac-glc
6′′-*O*-acetyl-genistin	H	OH	H	ac-glc
formononetin	Me	H	H	H
irilone	H	OH	OCH_2-	
prunetin	H	OH	H	Me
biochanin A	Me	OH	H	H
ononin	Me	H	H	glc
sissotrin	Me	OH	H	glc

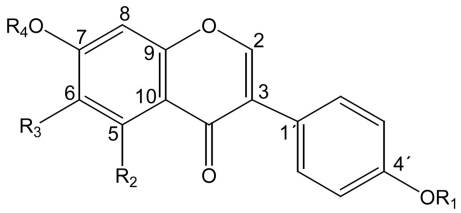

Figure 5. Structures of isolated isoflavones.

The remaining compounds in the other fractions are still not completely characterized. With the lyophilized coil residue it is possible to accomplish an HSCCC separation of the isoflavone aglycones. Purity of the isolated compounds has been verified by HPLC with a UV-detection at $\lambda = 260$ nm.

The structures of the isolated isoflavones are shown in Fig. 5.

4 Discussion

HSCCC has been applied to the preparative isolation of isoflavones from soy and red clover. The gentle operating conditions of HSCCC, in particular the lack of active surfaces, ensure a preparative isolation even of labile compounds. Using this separation technique, it is possible to obtain pure isoflavone standards or enriched isoflavone fractions with the latter ones being easily purified by HPLC. With these isolated isoflavones as standard material, we are now able to establish external calibration functions for the quantification of isoflavones in nutritional supplements. Moreover, it is possible to examine physiological effects, metabolism, and bioavailability of some rare isoflavones in more detail, such as, *e.g.* the methylenedioxy group containing red clover isoflavone irilone.

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