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Note

High-performance liquid chromatography of isoflavones and phytoalexins from *Cicer arietinum*

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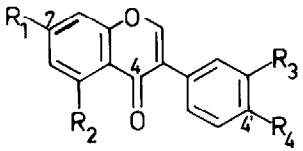
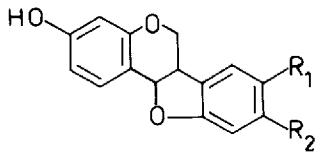
The main phenolic constituents of Bengalgram (*Cicer arietinum*) are the 7-O-glucoside 6″-malonates of the isoflavones formononetin (I) and biochanin A (II)[1]. These labile esters, which have also been detected in various other plants of the sub-family *Papilionoidae*[1,2], have until recently escaped detection owing to inadequate isolation procedures[3]. *Cicer arietinum* plants are also known to contain the isoflavones daidzein (III), genistein (IV) and pratensein (V)[5], together with the 7-O-glucosides of I and II (Fig. 1). Upon infection with phytopathogenic fungi the accumulation of the phytoalexins medicarpin (VI) and maackiain (VII) has also been observed in Bengalgram[6–8]. Both the isoflavones and the phytoalexins are of interest owing to their oestrogenic and antifungal properties[5,9].

Previous analyses of these phenols have used thin-layer chromatography, gas–liquid chromatography and gel filtration with subsequent spectrophotometric



(I) Formononetin: R_1 = OH; R_2 = R_3 = H; R_4 = OCH_3
(II) Biochanin A: R_1 = R_2 = OH; R_3 = H; R_4 = OCH_3
(III) Daidzein: R_1 = R_4 = OH; R_2 = R_3 = H
(IV) Genistein: R_1 = R_2 = R_4 = OH; R_3 = H
(V) Pratensein: R_1 = R_2 = R_3 = OH; R_4 = OCH_3



(VI) Medicarpin: R_1 = H; R_2 = OCH_3
(VII) Maackiain: R_1 = R_2 = O-CH_2-O

Fig. 1. Structures of isoflavones and phytoalexins isolated from *Cicer arietinum*.

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determination[3,8]. The recent high-performance liquid chromatographic (HPLC) separations of isoflavones[1,3,4,10,11] and a pterocarpan phytoalexin[12] have prompted us to develop a HPLC technique for the simultaneous separation of these two classes of Bengalgram constituents; this technique has meanwhile been employed in our studies on isoflavonoid metabolism in *Cicer arietinum* plants.

MATERIALS AND METHODS

The isoflavones and isoflavone 7-O-glucosides, as well as medicarpin and maackiain, were from the Institute's collection of natural products. Solvents were obtained from Merck (Darmstadt, F.R.G.) and Baker (Heidelberg, F.R.G.).

HPLC separations were carried out with a Kontron (Munich, F.R.G.) chromatograph (high-pressure pumps LC 410; gradient programmer, type 200; mixing chamber, type 810; UV/VIS detector, type LCD 725). Samples (20 μl) were separated using a LiChrosorb RP-8 or RP-18 column (250 × 4 mm I.D.; 5 μm) (Merck) and a flow-rate of 0.8 ml/min. A linear gradient of 20% B in (A + B) to 60% B in (A + B) in 35 min was applied. Solvent A was 3% acetic acid and Solvent B acetonitrile (LiChrosolv). Compounds were detected at 261 and 280 nm and quantitation (Shimadzu C-R1A integrator) was achieved by external standardisation.

Seeds of *Cicer arietinum* were obtained from local sources. To obtain samples of medicarpin and maackiain, seeds were germinated and treated as described by Keen[6]. Isolation of isoflavones and phytoalexins carried out by homogenising the plant material with acetone at −25°C and subsequent extraction (three to five times). The filtrate was concentrated at reduced pressure (max. 15°C) and brought to a defined volume before aliquots were directly used for HPLC analyses.

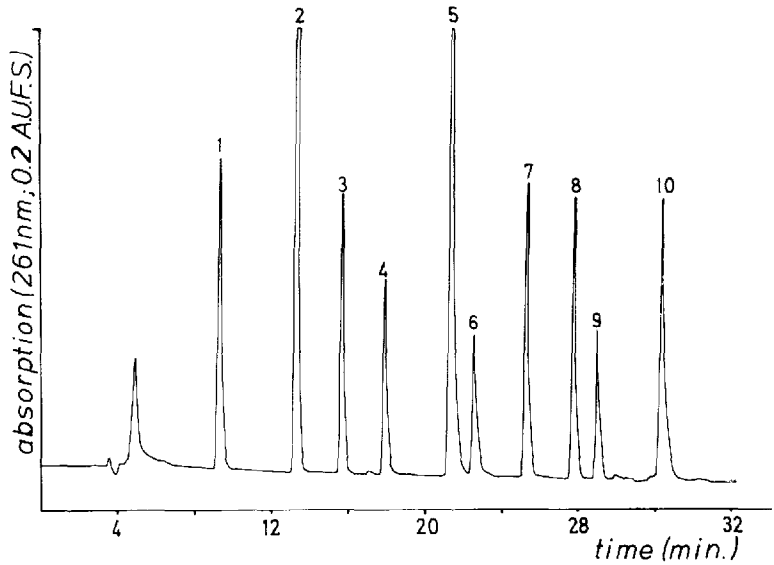

Fig. 2. HPLC chromatogram (RP-18 column with an acetic acid water acetonitrile gradient) of *Cicer arietinum* isoflavones and phytoalexins (standard mixture). Peaks: 1 = genistein 7-O-glucoside; 2 = formononetin 7-O-glucoside; 3 = daidzein; 4 = biochanin A 7-O-glucoside; 5 = genistein; 6 = pratensein; 7 = formonetin; 8 = maackiain; 9 = medicarpin; 10 = biochanin A.

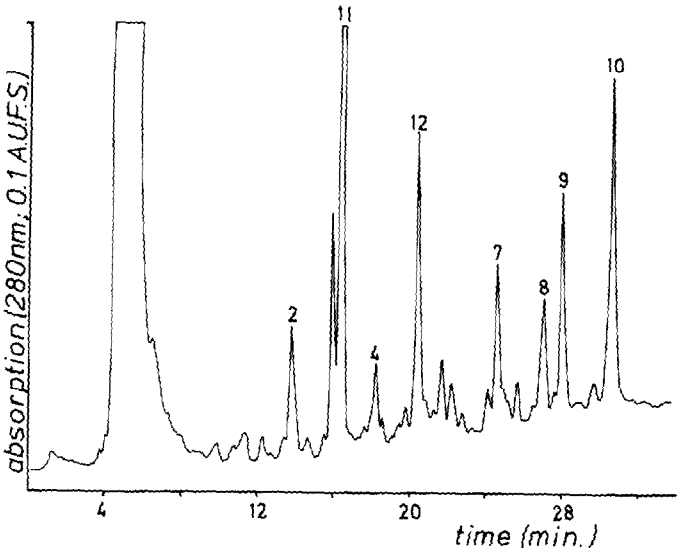

Fig. 3. HPLC chromatogram of fungus-infected *Cicer arietinum* seeds. Chromatography conditions and symbols 1–10 as in Fig. 2. Peaks: 11 = formononetin 7-O-glycoside 6″-malonate; 12 = biochanin A 7-O-glucoside 6″-malonate.

RESULTS AND DISCUSSION

Efficient separation of the phytoalexins VI and VII, together with the various isoflavones present in *Cicer arietinum* plants, was achieved by our chromatography system (Fig. 2). Compounds VI and VII appeared as sharp signals between the isoflavones formononetin (I) and biochanin A (II). The chromatogram further shows that a distinct separation of the 7-O-glucosides from the various aglycones could also be observed. This indicates that hydrolysis of isoflavone glucosides to isoflavone aglycones is not a prerequisite for a successful separation of isoflavone mixtures[11].

Extracts of fungus-infected *Cicer arietinum* plant material obtained according to Keen[6] were similarly chromatographed (Fig. 3). Compounds VI and VII could clearly be observed and, depending on the age of the infected plant material, the phytoalexins were measured in levels of 1.5–10 nmol/g fresh weight. Fig. 3 also documents the occurrence of I and II together with the 7-O-glucosides in these extracts. In contrast to our previous studies on non-infected Bengalgram[1], the 7-O-glucoside 6″-malonates of I and II no longer represent the bulk of the isoflavone material in these extracts. Endogenous hydrolysis of these malonyl conjugates to the 7-O-glucosides and the respective aglycones must have occurred.

In general, our HPLC system appears to be very suitable for the quantitative and simultaneous determination of the phytoalexins VI and VII, as well as the major isoflavones of fungus-challenged *Cicer arietinum* plants. Our present studies are devoted to determining the metabolic fate of the above-mentioned compounds in infected Bengalgram.

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