Journal of Chromatography, 267 (1983) 439–442
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,957

Note

High-performance liquid chromatography of isoflavonoid phytoalexins in French Bean (*Phaseolus vulgaris*)

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(Received April 28th, 1983)

Phytoalexins are low-molecular-weight antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to micro-organisms[1]. Therefore, they are believed to play a rôle in disease resistance in plants[2]. All phytoalexins accumulated in beans or other legumes are isoflavonoids. Especially in *Phaseolus vulgaris* L., a wide range of isoflavonoid phytoalexins is produced, *e.g.*, phaseollin, phaseollidin, phaseollinisoflavan, licoisoflavone A, kievitone and coumestrol (Fig. 1).

The relative amounts of these compounds depend on the type of micro-organism used as inducing agent[3], as well as on the incubation conditions during the accumulation period[4].

Up to now their isolation and quantification has been carried out by gas–liquid chromatography of the acetates[5] or by thin-layer chromatography (TLC). High-performance liquid chromatography (HPLC) has already been used succesfully for the separation of isoflavonoids from *Glycine max*[6–8] and *Cicer arietinum*[9]. Our work on the rôle of plant hormones in the phytoalexin interrelationship in *Phaseolus* required a rapid method of analysis of these compounds in different tissues. This report describes a method for rapid purification and quantification of isoflavonoid phytoalexins by HPLC, using a normal phase and gradient elution with isohydric solvents.

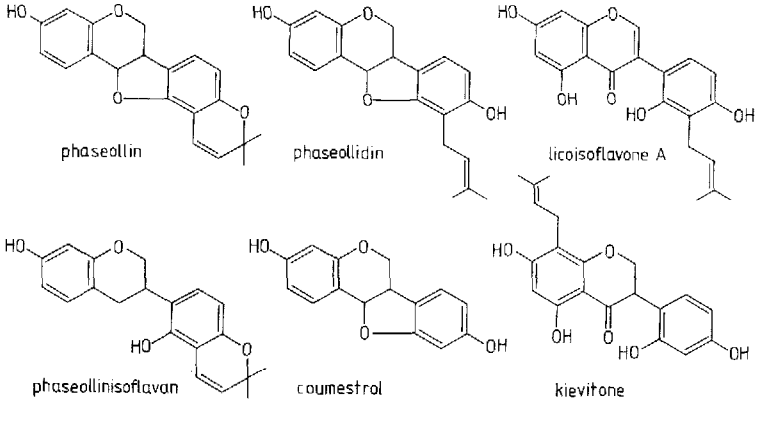

Fig. 1. Isoflavonoid phytoalexins in *Phaseolus vulgaris*.

0021-9673/83/$03.00

MATERIALS AND METHODS

Preparation of bean samples

Bean tissues were prepared and extracted as described elsewhere[4]. Prior to extraction, 100 μl vesidryl (2′,4,4′-trimethoxychalcone, 1 mg/ml) were added to each sample as an internal standard. After extraction the residue was redissolved in chloroform and a 25-μl sample was analysed by HPLC.

Isoflavonoid standards

Phaseollin and kievitone were purified from extracts of *Rhizoctonia solani*-infected bean hypocotyls following the method of Smith *et al.*[10]. Phaseollidin and licoisoflavone A were kindly provided by M. D. Woodward (University of Colorado, Boulder, CO, U.S.A.); phaseollinisoflavan was a gift from J. A. Bailey (Long Ashton Research Station, Bristol, Great Britain). Coumestrol was purchased from Eastman (Rochester, NY, U.S.A.). The internal standard vesidryl was synthesized following the cold condensation method of Geissman and Clinton[11].

Chromatography

The equipment was supplied by Waters Assoc. (Milford, MA, U.S.A.) and consisted of a M6000A and M45A Solvent Delivery System, a M660 Solvent Programmer, a U6K Universal Injector and a M40 Absorbance Detector with a 280-nm filter. A Hewlett-Packard 3390A Integrator was used for measuring peak areas.

The column (Lichroma, 0.3 cm I.D. × 30 cm) was packed with Lichrospher SI100 (10 μm) and eluted at 20°C with a linear gradient over 20 min (starting 1 min after injection), from 100% hexane–chloroform (2:1) to 100% chloroform–methanol

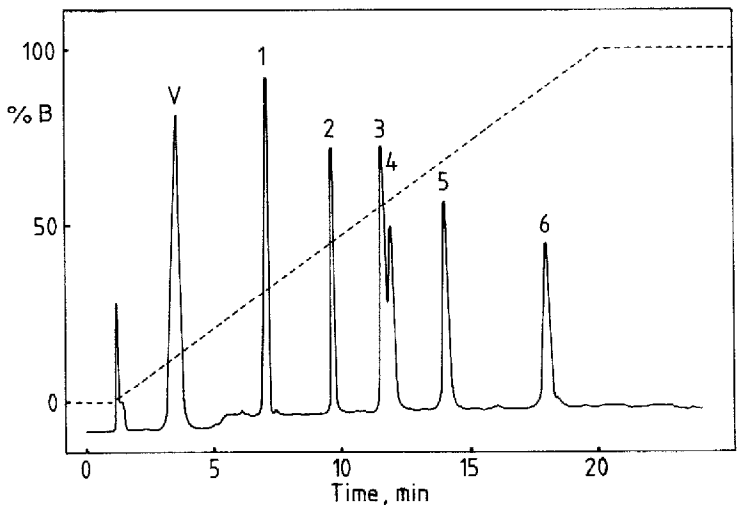

Fig. 2. Gradient elution of a mixture of isoflavonoid phytoalexin standards. Compounds: V = vesidryl; 1 = phaseollin; 2 = phaseollidin; 3 = phaseollinisoflavan; 4 = licoisoflavone; 5 = coumestrol; 6 = kievitone. Solvent A: hexane–chloroform (2:1). Solvent B: chloroform–methanol (100:8). The gradient as %B is represented by the broken line.

(100:8). The flow-rate was 2 ml/min. A guard-column (0.4 cm I.D. × 4 cm) packed with Spherisorb A (35–50 μm) was used to prevent deterioration of the main column.

Chloroform was purified by distillation (stabilized with 1% ethanol). Hexane was pre-washed with active charcoal to remove trace aromatics. Methanol was dried over a molecular sieve (Merck 5708) during 24 h, distilled and then 5.2% water was added to obtain an isohydric solvent[12].

RESULTS AND DISCUSSION

The chromatogram in Fig. 2 illustrates that in a gradient elution the six isoflavonoid phytoalexins were satisfactorily resolved. Only phaseollinisoflavan and licoisoflavone A were not completely separated. The position of vesidryl was of special importance with regard to its use as an internal standard. It eluted long enough after the solvent front and before the phytoalexins. Because of its chemical properties, similar to those of the phytoalexins, it could be used as internal standard to calculate extraction efficiencies (50–90% recovery). The time for one analysis was about 23 min, including re-equilibration to the initial conditions for 5 min.

This short re-equilibration time resulted from the use of isohydric methanol. By adding 5.2% water to dry methanol, the water content of the silica column remained constant during the gradient elution[12]. Therefore, the equilibration time between two runs and also retention time drift were greatly reduced. No water had to be added to the other solvents because of their already low water-holding capacity.

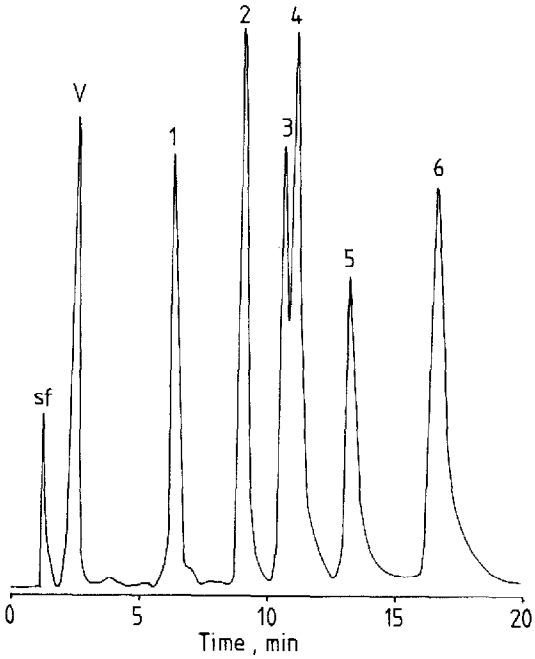

Fig. 3. Gradient elution profile of a mixture of isoflavonoid phytoalexin standards. Isohydric methanol in solvent B was substituted by dry methanol (0.1% water). Compounds as in Fig. 2. sf = Solvent front.

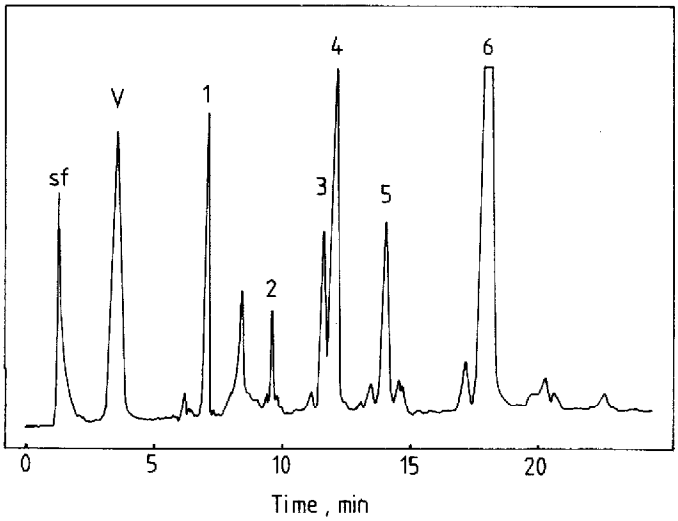

Fig. 4. Gradient elution of isoflavonoids in an ethanolic extract of bean cotyledons which received a phytoalexin-inducing treatment. Compounds as in Fig. 2. sf = Solvent front.

The use of isohydric methanol also improved peak shape (Fig. 3) and column life.

The elution pattern in Fig. 4 was obtained from an extract of bean cotyledons, which received a phytoalexin-inducing treatment. It shows that the six phytoalexins are present in this extract. This was confirmed by collecting the separate phytoalexins from several runs and identifying them by UV and mass spectra. The data obtained correspond to those reported previously[13,14].

This procedure makes large-scale TLC separations unnecessary. It is much faster and lowers the phytoalexin detection limit to 0.2 μg. With an internal standard, the method is accurate and useful for routine analysis.

ACKNOWLEDGEMENT

A. J. van Laere is a research associate of the Nationaal Fonds voor Wetenschappelijk Onderzoek (N.F.W.O.).

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