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Short Communication

Separation of aglucones, glucosides and prenylated isoflavones by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatography (HPLC) method was developed for the separation of lupin isoflavones, which consist of genistein and 2′-hydroxygenistein, their 7-O-glucosides and their respective 6-, 8-, 3′-mono- and 6,3′-diprenylated derivatives, as well as two coumaronochromones, lupinalbin A and B. HPLC was carried out with a Merck reversed-phase C_{18} LiChrospher 100 column using 45% solvent A (0.5% methanolic acetic acid) in 55% solvent B (0.5% aqueous acetic acid) for 2 min, followed by a gradient increase to 100% solvent A in 78 min then maintaining isocratic conditions for a further 10 min. The order of elution of the isoflavonoid derivatives was inversely proportional to their degree of polarity, with the 2′-hydroxygenistein glucoside eluting first and the 6,3′-diprenyl genistein eluting last.

INTRODUCTION

Simple isoflavones (Fig. 1; **1**, **1a**) are key intermediates in the biosynthesis of the isoflavonoid phytoalexins [1]. The latter are known to be synthesized *de novo* by members of the Leguminosae family in response to attack by microorganisms [2], or elicitation by biotic or abiotic elicitors [3]. In addition, several isoflavonoids have been reported to have antifungal [4], oestrogenic [5] and spasmolytic [6] activity and to be inducers of common *nod* genes in *Bradyrhizobium japonicum* [7] and inhibitors of the peroxidase-catalysed oxidation of lignin precursors [8].

Whereas most legumes accumulate simple isoflavones and their glucosides [9], white lupin (*Lupinus albus*) has a remarkable ability for the constitutive expression of a variety of prenylated isoflavones [10–12] as well as small amounts of several pyrano, dihydroxypyrano and dihydrofurano derivatives [13]. These compounds seem to replace the phytoalexins which commonly occur as antimicrobial defence compounds in many other legumes [2,3].

Previously used methods for the separation and determination of isoflavonoids are thin-layer and column liquid chromatography [14], or gas chromatography of the trimethylsilyl derivatives [15,16]. However, the poor resolution observed with the former procedures, and the need for derivatization in the latter, make these methods cumbersome. Fur-

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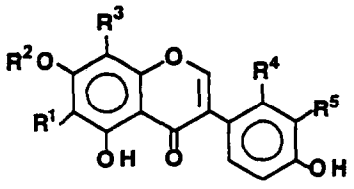


Isoflavonoids

1 R^1= R^2= R^3= R^4= R^5= H, genistein

1a R^1= R^2= R^3= R^5= H, R^4= OH, 2'-hydroxygenistein

2 R^1= R^3= R^4= R^5= H, R^2= glucosyl, genistein 7-O-glucoside

2a R^1= R^3= R^5= H, R^2= glucosyl, R^4= OH, 2'-hydroxygenistein 7-O-glucoside

3 R^1= CH^2CH=C(Me)^2, R^2= R^3= R^4= R^5= H, wighteone

3a R^1= CH_2CH=C(Me)$_2$, R^2= R^3= R^5= H, R^4= OH, luteone

4 R^1= R^2= R^4= R^5= H, R^3= CH_2CH=C(Me)$_2$, lupiwighteone

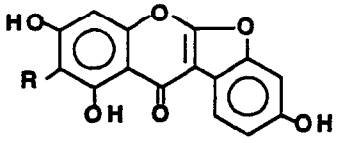
4a R^1= R^2= R^5= H, R^3= CH_2CH=C(Me)$_2$, R^4= OH, 2,3-dehydrokievitone

5 R^1= R^2= R^3= R^4= H, R^5= CH_2CH=C(Me)$_2$, isowighteone

5a R^1= R^2= R^3= H, R^4= OH, R^5= CH_2CH=C(Me)$_2$, licoisoflavone A

6 R^1= R^5= CH_2CH=C(Me)$_2$, R^2= R^3= R^4= H, lupalbigenin

6a R^1= R^5= CH_2CH=C(Me)$_2$, R^2= R^3= H, R^4= OH, 2'-hydroxylupalbigenin



Coumaronochromones

7 R= H, lupinalbin A

8 R= CH_2CH=C(Me)$_2$, lupinalbin B

Fig. 1. Isoflavonoid derivatives of white lupin. Me = Methyl.

thermore, the complexity of the isoflavonoid composition of some plant tissues, *e.g.* lupin roots [10–13], can require multiple chromatographic steps for their analysis and, therefore, the possibility of artifacts arising during fractionation and derivatization.

High-performance liquid chromatography (HPLC) has also been successfully applied to the determination of isoflavonoid compounds [14,17–23]. These compounds include the simple isoflavones, genistein and 2'-hydroxygenistein, and their 6-prenyl derivatives in white lupin hypocotyls [8]; genistein, daidzein and their glucosides in soybean (*Glycine max*) [17–21]; genistein, formononetin and

biochanin A in *Ononis spinosa* [22]; formononetin and biochanin A in chickpeas (*Cicer arietinum*) [23]; and the prenylated isoflavones jamaicin and lisetin in Jamaican dogwood (*Piscidia erythrina*) [24]. White lupin roots accumulate a complex mixture of isoflavonoids based on the aglucones genistein (**1**) and 2'-hydroygenistein (**1a**), their respective 7-O-glucosides (**2a**, **2b**), as well as the 6-, 8-, 3'-monoprenyl (**3**–**5**, **3a**–**5a**), 6,3'-diprenyl (**6**, **6a**) derivatives, and two related coumaronochromones (**7**, **8**) as major constituents [10–13] (Fig. 1). Such a variety of isoflavonoids with different substitution patterns and a wide range of polarity required the development of an HPLC protocol for their optimum separation, which is the subject of this paper.

EXPERIMENTAL

Chemicals

All isoflavonoid compounds were from our laboratory collection. These were previously isolated from white lupin roots and characterized by spectroscopic methods [10–13]. Chromatographic solvents were of analytical-reagent grade. They were passed through a 0.45-μm membrane filter (Type HA, Millipore) and degassed before use. All solvent ratios were on a volume basis.

Equipment

A Waters HPLC system (Millipore) was used equipped with an M45 solvent delivery system, and M441 absorbance detector (at 254 nm), a 7010 Rheodyne sample injector (20-μl loop) and an SIM interface module for data acquisition. Data were processed using Waters Baseline 810 software. Isoflavonoids were chromatographed on a Merck reversed-phase C_{18} LiChrosper 100 column, 250 × 4 mm I.D. (particle size 5 μm). Samples were passed through 3 mm diameter filters (pore size 22 μm) before injection through the column.

Extraction of isoflavonoids from tissue samples

Tissue extracts were prepared by homogenizing fresh filtered cells with three aliquots of aqueous 80% methanol (1:5, w/v) at room temperature. The combined methanolic extracts were evaporated *in vacuo* at 30°C to about 30% of their original volume and the aqueous residue was extracted twice with ethyl acetate (5:1, vol/tissue wt.). The organic layers were stirred with anhydrous sodium sulphate before being evaporated to dryness. The residue was dissolved in 80% aqueous methanol and stored at −20°C until analysed. This method gives an efficient extraction of the isoflavonoid aglucones, glucosides and prenylated derivatives, but not the malonylated glucosides which are usually soluble in 50% aqueous methanol [25,26].

Chromatography

For the determination of isoflavonoids in cell cultures, samples consisted of 20 μl in 80% aqueous methanol, which were equivalent to *ca.* 150 mg of fresh tissue. Chromatography was carried out at a flow-rate of 1 ml/min using 45% solvent A (0.5% methanolic acetic acid) in 55% solvent B (0.5% aqueous acetic acid) for 2 min. This was followed by a gradient increase to 100% solvent A in 78 min and isocratic conditions were maintained for a further 10 min. Equilibration of the column was achieved using 45% solvent A for 15 min before injection of a new sample. For quantitative analysis, known amounts of authentic compounds were injected to generate standard reference curves. Linearity was observed within a concentration range 10 ng to 5 μg of the reference compounds used (Fig. 2). Isoflavonoids in cell cultures were identified and determined using the software, based on their retention times

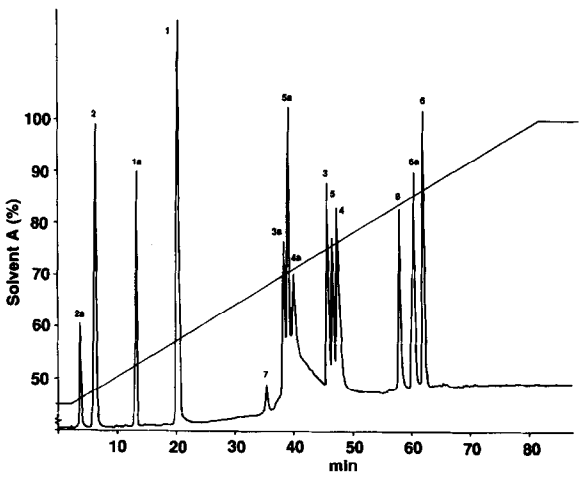


Fig. 2. HPLC profile for the elution pattern of authentic samples of the isoflavone (**1**–**6**, **1a**–**6a**) and coumaronochromone (**7**, **8**) derivatives (Fig. 1). Amounts of isoflavonoids injected were: 1 μg each of compounds **2a**, **4a** and **7**: 2 μg of **2**, **3**, **3a** and **8**; 2.5 μg of **1a**, **5** and **6a**; 3 μg of **4**, **5a** and **6**; 4 μg of **1**.

and the calibration graphs generated with the standard compounds, respectively. Whereas the retention times of isoflavonoids may vary slightly during different runs (*ca.* 0.05–0.5 min), they did not affect the relative positions of their peaks on the chromatogram.

RESULTS AND DISCUSSION

Separation of authentic isoflavonoids

The solvent gradient of choice was defined after extensive examination of various solvent combinations, flow-rates, and different linear and non-linear gradients. The elution procedure used gave an optimum and reproducible separation of the fourteen isoflavonoids of interst (Fig. 2). The inclusion of acetic acid in, and elimination of acetonitrile from, the mobile phase [24,27] allowed separation of the three monoprenylated isomers of genistein and 2′-hydroxygenistein (**3**–**5** and **3a**–**5a**, respectively) which could not otherwise have been resolved.

Several workers have used aqueous methanol in the separation of simple isoflavonoids [17–23], including some prenylated derivatives [8,24], but with limited success. However, this is the first report in which several isoflavonoid derivatives, with a wide range of polarity, have been resolved in a single chromatographic run (Fig. 2). As expected, the isoflavone glucosides (**2**, **2a**) eluted first, followed by the parent aglucones (**1**, **1a**) and then the monoprenylated (**3**–**5**, **3a**–**5a**) and diprenylated (**6**, **6a**) derivatives. Isoflavonoids prenylated at position 6 eluted first, followed by those prenylated at positions 3′, and 8. Whether they were glucosides, aglucones or prenylated compounds, the 2′-hydroxygenistein derivatives (**1a**–**6a**) eluted before those derived from genistein (**1**–**6**) and in the same relative order (Fig. 2). The coumaronochromones lupalbin A (**7**) and lupalbin B (**8**), however, had intermediate polarity and eluted before and just after the monoprenylated derivatives of genistein, respectively (Fig. 2).

Separation of isoflavonoids in lupin cell culture

The isoflavonoid profile of a cell culture derived from lupin radicle [28] is shown in Fig. 3. This profile shows all the major compounds known to occur in lupin roots [10–13], as well as a number of other unidentified peaks. The baseline drift observed during their separation is partly due to the increasing concentration of methanol in the gradient (Fig. 3), as well as the nature of the tissue analysed, especially for members of the Leguminosae family [21].

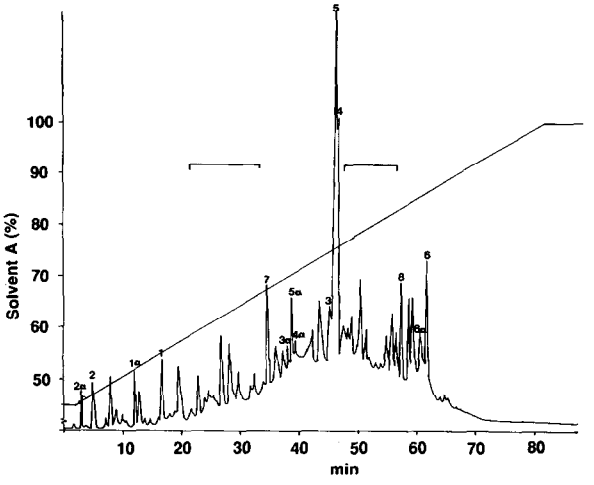


Fig. 3. HPLC profile for the elution pattern of isoflavonoid and coumaronochromone derivatives of a two-week old lupin cell suspension culture. Brackets indicate unidentified peaks.

TABLE I

AMOUNTS OF ISOFLAVONOIDS IN LUPIN CELLS AT DIFFERENT STAGES OF CULTURE GROWTH

Isoflavonoids[a]	Concentration of isoflavonoids (nmol/g fresh tissue)[b]		
	1-week culture	2-week culture	3-week culture
1	18.7	23.2	69.6
1a	16.9	33.1	132.5
2	85.2	68.6	12.8
2a	79.5	44.7	16.5
3	9.7	26.5	39.7
3a	8.6	12.5	37.6
4	38.5	60.3	167.3
4a	1.9	4.6	65.7
5	42.1	96.6	218.5
5a	6.9	12.8	66.7
6	15.6	49.9	89.6
6a	18.3	36.2	75.8
7	16.7	26.8	69.5
8	19.8	66.2	180.5

[a] Numbers correspond to compounds listed in Fig. 1.
[b] Average of two determinations with variations of 2–6%.

However, it is interesting to note the significant variations in the amounts of individual compounds accumulated within the cells, especially the decrease in the amount of glucosides and increased prenylated derivatives with culture growth (Table I). This clearly indicates the state of flux in metabolite production by cultured lupin cells, considering that a significant amount of the lipophilic, prenylated compounds (*ca.* 20%) is released into the nutrient culture medium [28]. In addition, several unidentified compounds (Fig. 3) were consistently observed in cell culture extracts and tend to increase in amount during culture growth. Their identity is currently being under investigation.

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