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Note

Quantitative analysis of the isoflavone phyto-oestrogens genistein, formononetin and biochanin A, in subterranean clover leaves by high-performance liquid chromatography

JOSEPH J. PATRONI*·*
Department of Organic Chemistry, University of Western Australia, Nedlands, W.A. 6009 (Australia)
and
W. J. COLLINS** and W. R. STERN
Department of Agronomy, Institute of Agriculture, University of Western Australia, Nedlands, W.A. 6009 (Australia)

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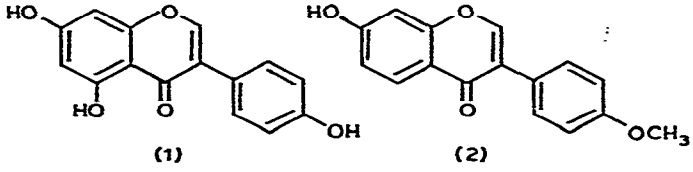
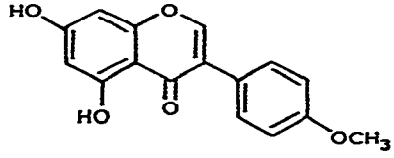
Oestrogenic activity in subterranean clover (*Trifolium subterraneum* L.) has been recognised for many years as a major cause in infertility problems in sheep[1]. Success in alleviating these problems has been due largely to the development, through breeding and selection, of lines of subterranean clover with low levels of oestrogenic activity. An essential part of this programme is the monitoring of levels of isoflavones on a large number of lines. Leaves of clover are assayed for the isoflavone phyto-oestrogens genistein (1), formononetin (2) and biochanin A (3). Originally, all three compounds were thought to be implicated in the infertility problem, but there is now strong evidence to suggest that formononetin alone, is the main factor involved[2].



(1)

(2)



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* Present address: Sugar Research Institute, Mackay, Queensland 4740, Australia.
** Present address: Western Australian Department of Agriculture, South Perth, W.A. 6151, Australia.

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The analytical method used in the isoflavone laboratory is based on the visual estimation of developed thin-layer chromatographic plates[3]. The method is semi-quantitative and was developed from the chromatographic method of Beck[4] and an extraction technique modified from that of Curnow[5].

It is some years since an accurate analysis of the levels of isoflavones in subterranean clover was undertaken. For this purpose we used high-performance liquid chromatographic (HPLC) procedures. The high ultraviolet absorption (λ_{max} 263–264 nm), coupled with the moderately polar nature of these isoflavones were considered ideal characteristics for the application of UV-monitored C_{18} reversed-phase chromatography with a polar eluent such as methanol–water.

This paper describes the successful resolution and quantitation of the isoflavones 1, 2 and 3 found in fresh clover leaves.

EXPERIMENTAL

Materials and apparatus

All solvents were analytical-reagent grade.

HPLC was with a Waters Assoc. (Milford, MA, U.S.A.) M-6000A pump fitted with a μBondapak™ C_{18} (30 cm × 3.9 mm I.D.) pre-packed column and a U6K loop injector. Detection was by elution monitoring with a Waters 440 absorbance detector (254 nm, 0.5 a.u.f.s.). Output was measured on a Hewlett-Packard 3380A integrator using the external-standard area quantitation method. Manual injection was with a Hamilton 800 (25 μl) syringe with the chromatographic system at ambient temperature.

Two standard solutions consisting of a mixture of isoflavones 1, 2 and 3 in methanol (250 μg/ml and 50 μg/ml) were stored at 2°C for a period of 3 months without decomposition.

Procedure

Twelve circular clips of undamaged subterranean clover leaves were ground with a glass rod and acid-washed sand (0.1 g). Ethanol (1 ml) was then added and the mixture allowed to stand in a water bath (60°C, 10 min). The extract was decanted and the extraction procedure repeated on the residue to give a combined total extract (2.0 ml) as described previously[3]. Part of the solution (1.0 ml) was then evaporated to 0.5 ml and applied to Sep-Pak™ C_{18} cartridges, pre-washed with methanol, and finally eluted with methanol (4 × 1 ml). This eluent was concentrated to dryness (water bath at 60°C) and made up to a final volume with methanol (2.0 ml).

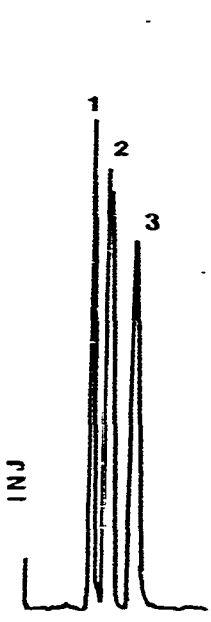
Reversed-phase HPLC [methanol–water (27:73) at a flow-rate of 2.5 ml/min, 2500 p.s.i.] of the prepared extract (20-μl injections), gave symmetrical peaks with baseline resolution of the isoflavones 1, 2 and 3. Detector response area was shown to be linear over the relevant range (0.5–5.0 μg) for each of the three standards. Quantitation was therefore determined by calibration of the on-line integrator by an external-standard mixture prior to analytical runs. Reproducibility of quantitation of a standard mixture of 1, 2 and 3 subjected to this analytical procedure was within 5% at the 1-μg level.

RESULTS AND DISCUSSION

The system described allowed analysis of some 1000 clover-leaf extracts with negligible loss of column performance.

Typical chromatograms obtained from standard solution (Fig. 1) and from extract (Fig. 2) gave symmetrical, well-resolved peaks. Over a period of 3 months retention times of compounds 1, 2 and 3 varied only slightly and within the ranges 1.52–1.59, 1.82–1.96, 2.26–2.57 min, respectively.

The levels of isoflavones determined for a range of subterranean clover cultivars will be reported in a separate publication.



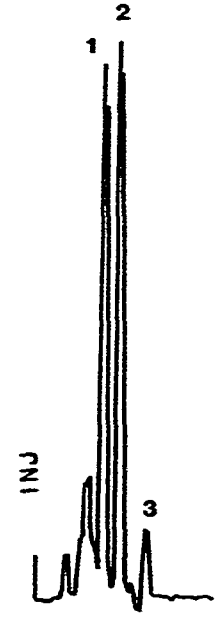


Fig. 1. HPLC separation of a standard mixture of genistein (1) (996 ng), formononetin (2) (1008 ng), and biochanin A (3) (1024 ng).

Fig. 2. HPLC separation of a typical clover leaf extract. Peaks: 1 = genistein (1040 ng); 2 = formononetin (1110 ng); 3 = biochanin A (180 ng). Chromatographic conditions are described in the Experimental section.

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REFERENCES

1 G. R. Moule, A. W. H. Braden and D. R. Lamond, *Anim. Breed. Abst.*, 31 (1963) 139.
2 A. W. H. Braden and D. A. Shutt, in M. J. T. Norman (Editor), *Proceedings of the XIth International Grassland Congress, 1970, Surfers Paradise, Australia*, University of Queensland Press, 1970, p. 770.
3 C. M. Francis and A. J. Millington, *Aust. J. Agr. Res.*, 16 (1965) 557.
4 A. B. Beck, *Aust. J. Agr. Res.*, 15 (1964) 223.
5 D. H. Curnow, *Biochem. J.*, 58 (1954) 283.