Journal of Chromatography, 346 (1985) 456–460
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 986

Note

Reversed-phase high-performance liquid chromatographic separation of closely related furocoumarins

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(Received July 1st, 1985)

Furocoumarins are known to exhibit several biological effects, most prominent of which are their photosensitizing and the mutagenic activities[1–4]. Many plant species contain furocoumarins, and these potently active and toxic substances necessitate efficient analytical methods for their qualitative and quantitative determination.

In the last few years, high-performance liquid chromatography (HPLC) has been used increasingly for furocoumarin separations and determinations. It was shown to be a rapid and sensitive method for the detection of phototoxic psoralens in Citrus essential oils[5–9]. Such oils are commonly used in the fragrance and flavour industry. The application of HPLC to the determination of the strong photosensitizer bergapten in perfumes and suntan cosmetics has also been reported[10,11]. Of special interest is the HPLC determination of 8-methoxypsoralen plasma levels in conjunction with photochemotherapy in the treatment of psoriasis[12–14]. Furthermore, HPLC has proven useful for the investigation of plants containing furocoumarins[14–19]; these very complex mixtures require efficient separation methods. Two recent publications have dealt with the separation of a large number of natural and synthetic coumarins[20,21]. Van de Casteele *et al.*[20] separated 43 coumarins including 6 furocoumarins using a combination of isocratic and linear gradient systems on a RP-18 column. Thompson and Brown[21] studied the behaviour of 67 coumarins (including 19 furocoumarins) on normal phase silica and on RP-18 columns and found that the two types of columns can complement each other.

In this paper a reversed-phase separation system for closely related furocoumarins is presented. These compounds (Fig. 1) occur in several Umbelliferae genera[22], *e.g.*, *Heracleum*, *Angelica* and *Peucedanum*, and show very similar chromatographic behaviours. For improvement of the peak identification, the HPLC system was equipped with a photodiode array detector[23] which has already been shown to be helpful in the HPLC of flavonoids[24,25], xanthones[24] and iridoids[25].

MATERIALS AND METHODS

All solvents were of HPLC quality, purchased from Fluka (Buchs, Switzerland). HPLC separations were carried out with a Waters gradient system consisting of two M 6000 A pumps, a Model 720 system controller and a WISP 710 B autosampler. For UV detection a 1040 A high speed spectrophotometric detector (Hew-

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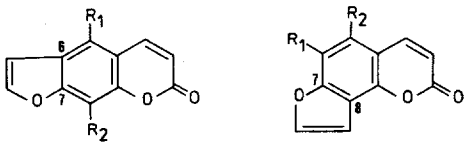

R_1	R_2		R_1	R_2	
H	H	Psoralen	H	H	Angelicin
OCH_3	H	Bergapten	OCH_3	H	Sphondin
H	OCH_3	8-Methoxypsoralen	H	OCH_3	Iso-Bergapten
OCH_3	OCH_3	Iso-Pimpinellin	OCH_3	OCH_3	Pimpinellin

Fig. 1. Structures of investigated furocoumarins.

lett-Packard, Waldbronn, F.R.G.) was used at 310 nm. The gradient separations were carried out using a Knauer Spherisorb ODS II (3 μm) cartridge column (100 × 4 mm I.D.). Solvent A was tetrahydrofuran–water (3.5:96.5) and solvent B was methanol. The gradient profile was as follows: 0–20 min, 25% B in A (isocratic); 20–40 min, 25–45% B in A (linear gradient). The flow-rate was 1.0 ml/min.

Bergapten, isobergapten, pimpinellin, isopimpinellin and sphondin were isolated from *Heracleum sphondylium* roots using centrifugally accelerated thin-layer chromatography (TLC). Angelicin and psoralen were synthesized[26] and isolated from the reaction mixture by centrifugally accelerated TLC. 8-Methoxypsoralen was obtained from Fluka. The reference sample concentration for HPLC was about 0.1 mg/ml in methanol and the injection volume 10 μl.

The dried plant material was extracted with chloroform. For analyses, the crude extracts were dissolved in methanol at a concentration of 10 mg/ml and prepurified over Bond-Elut C_{18} cartridges (Analytichem Intern., Habor City, CA, U.S.A.). *Heracleum sphondylium* roots were obtained from Dixa (St. Gallen, Switzerland) and *Heracleum mantegazzianum* leaves were collected in Germany.

RESULTS AND DISCUSSION

An efficient separation of the eight furocoumarins could be achieved with the described HPLC system (Fig. 2). From the obtained results it is supposed that the use of a small (3 μm) spherical octadecylsilane packing material represents a significant improvement for HPLC separations of such compounds of low polarity. The peak shape and symmetry observed is improved in comparison with those found on the 10-μm irregular ODS material. As the compounds lie in a narrow polarity range the analysis time must be kept relatively long. At higher solvent strengths the resolution between the first three peaks becomes unsatisfactory. The mobile phase flow-rate cannot be increased since the column pressure is 4000 p.s.i. with the solvent system used. The first three components eluted were difficult to separate. A sufficient mobile phase selectivity was obtained by addition of tetrahydrofuran: higher or lower amounts of tetrahydrofuran than that added impaired the separation of the former group of furocoumarins.

In spite of the retention time variation, a certain peak identification is nevertheless possible by use of the photodiode array detector due to the characteristic UV spectra of each of the components. The gradient system has proven suitable for the

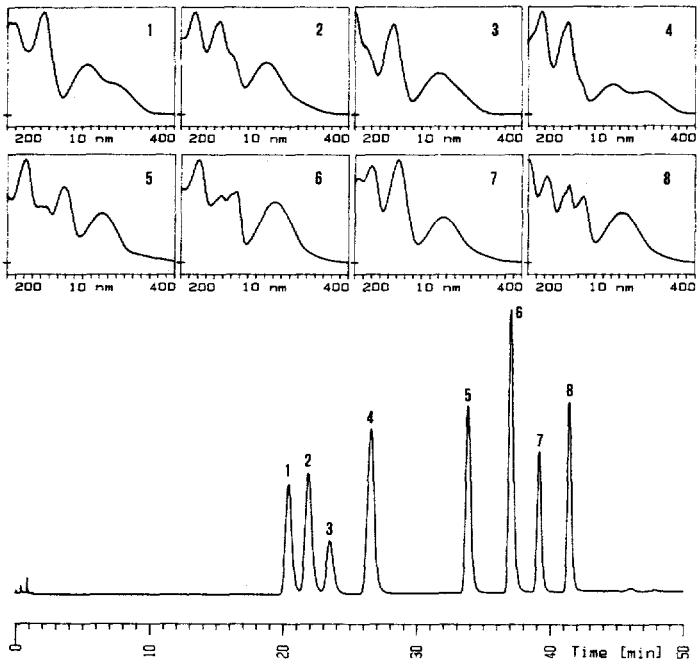

Fig. 2. Gradient HPLC separation of furocoumarin references with on-line UV detection. Column: Knauer Spherisorb ODS II cartridge, 3 μm, 100 × 4 mm I.D. For gradient profile see text. Peaks: 1 = psoralen; 2 = 8-methoxypsoralen; 3 = angelicin; 4 = sphondin; 5 = isopimpinellin; 6 = bergapten; 7 = pimpinellin; 8 = isobergapten.

analysis of plant extracts (Figs. 3 and 4). From the chromatograms it is seen that *Heracleum sphondylium* roots contain predominantly the compounds 4–8 (Fig. 3), whole in *Heracleum mantegazzianum* leaves the first eluting compounds 1–3 and 6 (Fig. 4) represent the main furocoumarins. The photodiode array detector allows an unambiguous identification of even low amounts of coumarins in the extracts. UV spectra over the range 200–400 nm can be compared with reference spectra (attenuation > 8 m.a.u.).

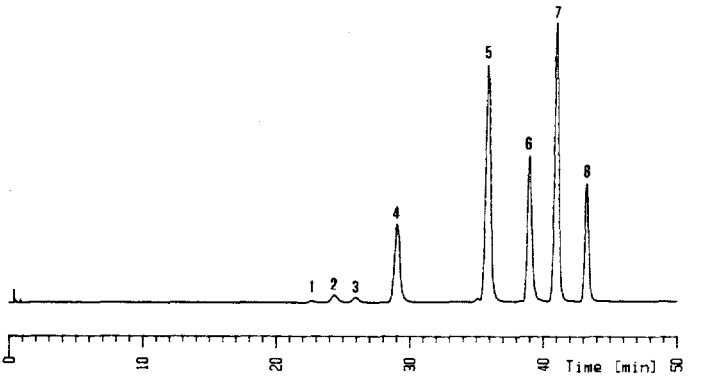

Fig. 3. Gradient HPLC separation of *Heracleum sphondylium* root furocoumarins. For conditions see Fig. 2 and text. All peaks were identified by on-line UV detection between 200 and 400 nm.

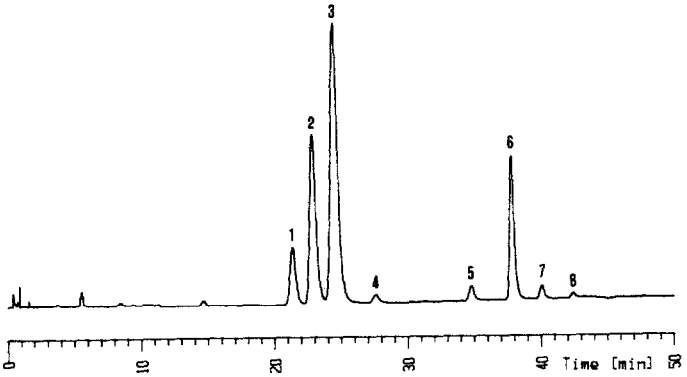

Fig. 4. Gradient HPLC separation of *Heracleum mantegazzianum* leaf furocoumarins. For conditions see Fig. 2 and text. All peaks were identified by on-line UV detection between 200 and 400 nm.

As these furocoumarins are widespread in some plant families, they could serve as chemotaxonomic markers. This is of importance, *e.g.*, for the Umbelliferae family, since many of the species are botanically not easy to define or to distinguish. The presented HPLC gradient system may be appropriate for such a chemotaxonomic investigation as the two plant examples given indicate. The clean-up procedure for the crude extracts is very easy and efficient, and the automated HPLC system allows continuous analyses. The amount of plant material required for an analysis is about several hundred milligrams, depending on the furocoumarin content of the plant. Further work will be done to examine the furocoumarin contents of various plants and pharmaceutical preparations, together with the phototoxic and photomutagenic activities.

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