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Note

The use of new chromatographic techniques for the isolation and purification of phenolic acids from *Indigofera heterantha*

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Although standard procedures based on thin-layer, paper and column chromatography still play a major rôle in the isolation and purification of plant phenolics[1,2], the development of numerous new separation techniques has considerably increased the speed of separation and degree of resolution. The new technique available today to plant chemists include mainly high-performance liquid chromatography (HPLC), centrifugal thin-layer chromatography (CTLC), flash and vacuum liquid chromatography (VLC). HPLC is now a well established technique and has proved its usefulness in the separation of numerous types of phenolics[1,3–5]. CTLC also known as Circular Chromatotron has been successfully used for the isolation and purification of various coumarins[6,7] and Xanthones[8]. Flash liquid chromatography and VLC are two rapid techniques[9,10] which are particularly useful for fractionation of crude plant extracts. However it should be noted that all these chromatographic methods are not mutually exclusive, but complementary. Pure compounds from crude plant extracts can be obtained rapidly only by combining the various chromatographic techniques available.

Phytochemical studies of Indigofera species have shown the presence of various phenolic compounds[11–13]. In the present work, attempts have been made to isolate and purify rapidly phenolic acids from leaves and flowers of *Indigofera heterantha* (Leguminosae) by the use of newly developed chromatographic techniques.

EXPERIMENTAL

Plant material

Branches bearing leaves and flowers of *Indigofera heterantha* were collected from Murree Hills (Pakistan) in mid-May 1988.

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Extraction

The phenolic acids were extracted from air-dried leaves and flowers of *I. heterantha* by acid hydrolysis of the plant material, using the standard method of hydrolysis[14].

Isolation and purification

The following chromatographic techniques were employed for the isolation and purification of phenolic acids.

Vacuum liquid chromatography (VLC). The methanolic leaf extract was deposited on top of a TLC grade polyamide column. Elution under vacuum was carried out with benzene progressively enriched with methyl ethyl ketone and methanol. Twenty six fractions were collected in 2 h. Fractions 4 and 5 contained a single pure phenolic acid (V'), violet in colour. Another bluish violet constituent (BV') was the main compound of fractions 6 and 7 along with V'. A third blue compound (B') along with a flavonic aglycone was present in fraction 13.

Column chromatography. All the three constituents of the leaf extract, *i.e.*, V', BV' and B' were allowed to percolate rapidly through a narrow polyamide column, using the same eluting solvents as for VLC.

Thin-layer chromatography (TLC). The purity of each compound was monitored on TLC plates in four solvent systems. Each phenolic acid appeared as a single pure spot in all the systems employed. Their R_F values were noted and are presented in Table I.

High-performance liquid chromatography (HPLC). In order to check finally the purity of the isolated phenolic acids, HPLC analysis was carried out. A Shimadzu HPLC LC-6A equipped with an UV detector and an auto injector was used. Though each of the phenolic acids appeared pure on TLC plates, HPLC analysis revealed the presence of impurities in each sample (Fig. 1). Moreover, it was observed that BV' still contained V'. Using a larger HPLC column it was possible to purify all the three acids by semi-preparative HPLC (Fig. 2).

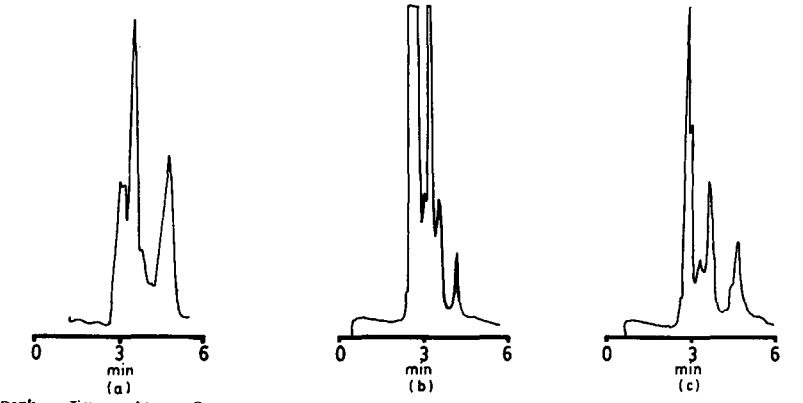
Flash chromatography. A methanolic flower extract of *I. heterantha* was deposited on top of a column-grade polyamide packed column, fitted with a solvent reservoir and a valve through which oxygen-free nitrogen was allowed to flow through the column. The flow-rate of the eluting solvents, chloroform, chloroform–methanol and

TABLE I

CHROMATOGRAPHIC AND UV SPECTRAL DATA OF PHENOLIC ACIDS IN *I. HETERANTHA*

Solvents on silica gel: 1 = acetic acid–chloroform (1:9); 2 = ethyl acetate–benzene (9:11). Solvents on cellulose: 3 = benzene–methanol–acetic acid (45:8:4); 4 = 6% aqueous acetic acid.

Compound	*TLC R_F (·100) in solvents*				*λ_{max} (nm)*		*Colour under UV*
	1	*2*	*3*	*4*	*Ethanol*	*Ethanol–sodium hydroxide*	
BV'	48	65	93	31	290	280	Blue-violet
V'	42	67	93	36	278, 314	300	Violet
B'	37	44	87	59	237, 332	312	Blue



Peak	Time	Area	Conc.
1	2.062	742351	22.3539
2	2.540	1181836	35.5878
3	2.920	238775	7.1900
4	3.998	1157943	34.8683

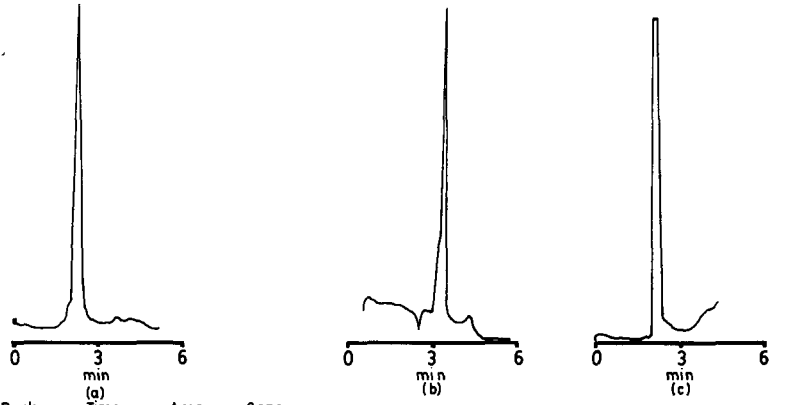
Peak	Time	Area	Conc.
1	0.298	75533	1.2621
2	2.415	4150426	69.3479
3	3.152	1204403	20.1239
4	3.520	347556	5.8072
5	4.270	207019	3.4590

Peak	Time	Area	Conc.
1	2.212	53474	2.2290
2	2.417	997150	41.5658
3	2.990	225515	9.4005
4	3.317	597274	24.8971
5	4.498	476821	19.8761
6	5.298	48734	2.0315

Fig. 1. HPLC analysis of (a) B′, (b) BV′, (c) V′. Column: Shim-pack CLC-ODS (0.15 m × 6.0 mm I.D.). Solvent: methanol–water–acetic acid (75:24:1). Flow-rate: 1.2 ml/min. Detection: fixed UV at 280 nm. Temperature: ambient.

methanol, was controlled by the flow valve. The ten fractions, which were collected under UV light at 366 nm in less than half an hour contained mainly flavonoid aglycones. However, fraction 1 was a equivalent mixture of two phenolic acids.

Centrifugal thin-layer chromatography (CTLC). Fraction 1 was applied to a 2 mm thick silica gel PF 254 rotor of a Circular Chromatotron Model 7924. The phenolic acids were eluted with chloroform enriched progressively with acetic acid. Two concentric bands PA1 and PA2 that separated by centrifugal force and under an inert



Peak	Time	Area	Conc.
1	2.613	105033	100

Peak	Time	Area	Conc.
1	3.20	41601	100

Peak	Time	Area	Conc.
1	2.423	135037	100

Fig. 2. Semi-preparative purification of (a) B′, (b) BV′, (c) V′. Column: Ultrasphere ODS (25 cm × 4.6 mm I.D.). Other conditions as in Fig. 1.

atmosphere were observed by UV light at 254 nm and were collected in separate sample tubes. Both these acids, shown to be very pure by HPLC, were co-chromatographed with the three phenolic acids obtained from leaves of *I. heterantha*. It was noted that PA1 was identical to BV′ and PA2 to B′.

On the basis of their chromatographic behaviour, colour under UV light and UV spectral data in ethanol and ethanol–sodium hydroxide (Table I), the three phenolic acids BV′, B′ and V′ were identified as protocatechuic, genestic and *p*-methoxycinnamic acids respectively.

CONCLUSIONS

The combined use of new as well as conventional chromatographic techniques is quite effective for the speedy isolation and purification of phenolic acids. The much shorter and increased resolution can improve the analysis of many more phenolic constituents.

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