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Determination of the norlignan glucosides of Hypoxidaceae by high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatography system with UV detection is proposed for the qualitative and quantitative determination of norlignan glucosides isolated from some African Hypoxidaceae used in traditional medicine and, more recently, for pharmaceutical preparations. The analysis indicated the occurrence of these compounds, the aglucones of which are characterized by the rare C_6H_5–C_5–C_6H_5 skeleton type, in other Hypoxidaceae of different geographical origin.

INTRODUCTION

Hypoxidaceae were first considered as distinct from Amaryllidaceae in 1814 [1] and after some hesitation were eventually identified as a separate family in 1964 [2]. The family includes many genera such as *Hypoxis, Curculigo, Pauridia, Molinera, Spiloxene, Empodium, Rhodohypoxis, Campynema* and *Capynemanthe,* which are found in the southern hemisphere and in southern Africa in particular. According to the tradition of the local medicine, the rhizomes of the genus *Hypoxis* are prescribed for prostatic hypertrophy and internal cancer [3]. The former indication, for which a phytopharmaceutical is marketed in Germany [4], has been associated with chemically non-defined steroid glucosides, whereas the latter (structures presented in Fig. 1) is related to glucosides of unsaturated aglucones which, on the basis of structural evidence and biogenetic considerations, can be considered as norlignans. These compounds have the rare skeleton type C_6H_5–C_5–C_6H_5, 1,3- or 1,5-diphenylpentane. To the latter series belong the first diglucoside isolated, hypoxoside, **1** [5], the two monoglucosides of its aglucone, rooperol (**2**),obtuside A (**3**) and obtuside B (**4**) [6], isolated from *Hypoxis obtusa* of Mozambique, and nyasicoside (**5**), isolated from *Hypoxis nyasica* of Malawi [7] and *Curculigo recurvata* of Zaire [8]. Interjectin (**6**), a complex diglucoside of nyasicol, the aglucone of nyasicoside, has recently been isolated from *Hypoxis interjecta* and *Hypoxis multiceps* of South Africa [9]. Two other glucosides, curculigine and hypoxine, isolated from *Curculigo recurvata* and *Hyposix obtusa,* respectively, from Mozambique are currently under study.

Nyasoside (**7**) [3], the two monoglucosides of its aglucone, mononyasine A (**8**) and mononyasine B (**9**) [10] and the triglucoside nyaside (**10**) [11], isolated with hypoxoside and nyasicoside from *Hypoxis nyasica* of Malawi belong to the 1,3-diphenylpentane series. The aglucone of nyasoside, nyasol (**11**), was isolated from *Hypoxis angustifolia* from Zimbabwe [12].

As a result of the promising cytotoxic and antitumour activities of these compounds, some of which have been patented [13,14], research on the *in vitro* propagation of rhizomes and unopened flower buds is in progress [15,16]; the seeds are not capable of vegetal propagation.

The two main glucosides, hypoxoside and nyaso-

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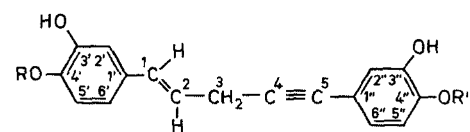
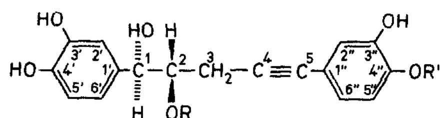
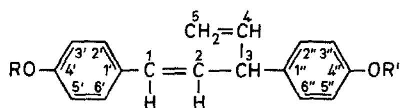


	R	R'	
1	Gl	Gl	hypoxoside
2	H	H	rooperol
3	Gl	H	obtuside A
4	H	Gl	obtuside B



	R	R'	
5	Gl	H	nyasicoside
6	p-hydroxycinnamoyl →2-Gl	Gl	interjectin



	R	R'	
7	Gl	Gl	nyasoside
8	H	Gl	mononyasine A
9	Gl	H	mononyasine B
10	Gl	Ap→6-Gl	nyaside
11	H	H	nyasol

Fig. 1. Structures of nortignan glucosides of nypoxidaceae.

side, can be separated with difficulty by counter-current distribution, thin-layer chromatography [3] and by high-performance liquid chromatography (HPLC) [16]. The method proposed here can separate all known glucosides except the isomer mono-glucosides **3**, **4**, **8** and **9**, using a reversed-phase column with an acidic eluent (pH 3.0). It is useful for monitoring production in tissue cultures and for establishing the presence of these norlignan glucosides, which can be of taxonomical relevance for the genus *Hypoxis* and other genera of Hypoxidaceae.

EXPERIMENTAL

Plant material

The samples examined were: rhizomes of *Hypoxis obtusa* Burch from Maputo (Mozambique); rhizomes of *Hypoxis obtusa* Burch-complex supplied by Dr. S. Sibanda, Harare (Zimbabwe) and by Dr. E. Nyandat, Nairobi (Kenya); rhizomes of *Hypoxis angustifolia* Lam. supplied by Dr. P. Rasoanaivo, Antananarivo (Madagascar) and by Dr. S. Sibanda, Harare (Zimbabwe); rhizomes of *Hypoxis dubumbens* Lin. supplied by Dr. A. Chiappeta, Pernanbuco (Brazil); rhizomes of *Hypoxis nyasica* Bak. supplied by Professor J. D. Msonthi, Zomba (Malawi); rhizomes of *Hypoxis interjecta* Nel, Pretoria (South Africa), *Hypoxis multiceps* Buching ex Krauss, Pretoria (South Africa), *Hypoxis argentea*, Pretoria (South Africa) and *Campynema lineare*, Tasmania, obtained from Professor P. Raven (Director of the Missouri Botanical Garden); and rhizomes and flowers of *Curculigo recurvata* Dryand obtained from Dr. Chifundera Kusamba (Zaire).

Reagents

The chemicals used were of analytical reagent-grade. The acetonitrile used was of HPLC grade. Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

TABLE I

RETENTION TIMES AND CAPACITY FACTORS

Compound	Retention time (min)	Capacity factor
Curculigine	13.28	2.30
5	14.43	2.58
Hypoxine	18.02	3.44
6	18.57	3.61
1	22.37	4.55
10	22.98	4.70
7	23.96	4.96
3 and **4**	25.12	5.24
2	27.97	5.94
8 and **9**	29.67	6.36
11	35.62	7.84

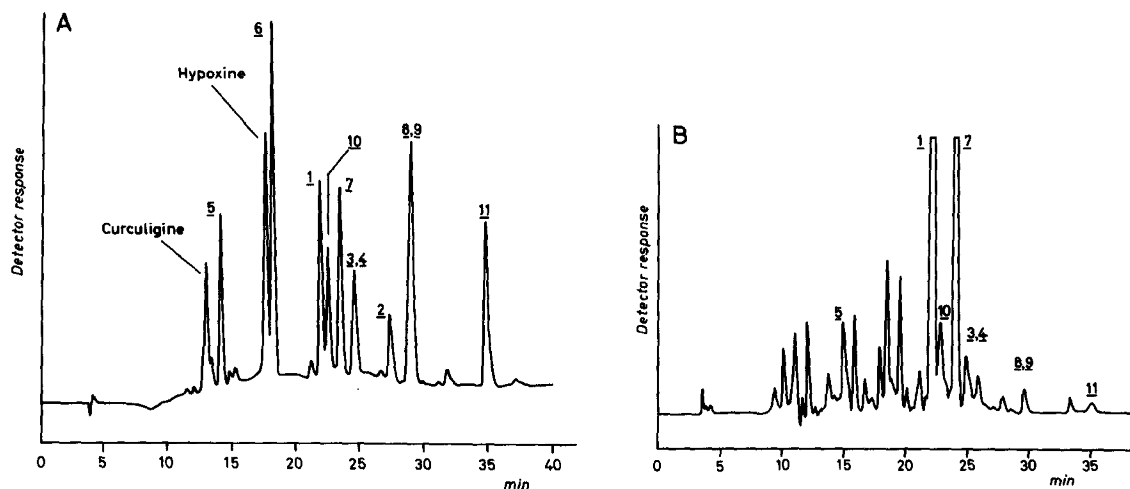


Fig. 2. (A) Chromatogram of a mixture of reference compounds. Peaks: curculigine; 5 = nyasicoside; hypoxine; 6 = interjectin; 1 = hypoxoside; 10 = nyaside; 7 = nyasoside; 3 = obtuside A; 4 = obtuside B; 2 = rooperol; 8 = mononyasine A; 9 = mononyasine B; 11 = nyasol. (B) Chromatogram of the methanol extract from *Hypoxis obtusa* rhizomes of Zimbabwe. The chromatographic conditions are given under Experimental.

Instrumentation

The HPLC analyses were performed on a Perkin-Elmer system (Norwalk, CT, USA) equipped with a Series 3 liquid chromatograph connected to an LC-75 spectrophotometric detector. The samples were introduced with an injection valve Rheodyne 7125 (Berkeley, CA, USA) with a 100-μl loop. Chromatograms were recorded on a Perkin-Elmer Sigma 10 B chromatography data station. The column was 250 × 4.6 mm I.D. (5 μm particle size) Spherisorb ODS-2 (Phase Separations, Queensferry, UK).

High-performance liquid chromatography

The separations were achieved using a linear gradient of acetonitrile (solvent A) and 0.05 *M* phosphate buffer at pH 3.0 (solvent B), increasing from 20% solvent A at time t = 0 min to 70% solvent A at t = 30 min with a constant flow-rate of 0.8 ml/min at room temperature. The eluent was monitored at 260 nm.

Preparation of natural standards

Hypoxoxide, nyasoside, obtusides A and B, mononyasines A and B, nyaside, nyasicoside, inter-

TABLE II

LINEARITY RESPONSE AS A FUNCTION OF CONCENTRATION

y = peak area; x = amount (μg per injection).

Compound	Range tested (μg)	Regression equation	Correlation coefficient
5	0.88–7.04	$y = 1.182x - 0.263$	0.9989
6	0.30–4.80	$y = 0.978x - 0.082$	0.9997
1	0.15–2.32	$y = 4.732x - 0.078$	0.9998
10	0.60–9.60	$y = 1.497x - 0.008$	0.9979
7	0.55–8.80	$y = 1.312x - 0.018$	0.9996
3 and **4**	0.90–14.40	$y = 2.744x - 0.013$	0.9987
2	0.46–7.36	$y = 2.499x - 0.037$	0.9993
8 and **9**	0.17–2.72	$y = 5.634x - 0.090$	0.9992
11	0.20–8.00	$y = 1.786x - 0.025$	0.9997

TABLE III

QUALITATIVE AND QUANTITATIVE ANALYSIS OF THE METHANOLIC EXTRACTS

Plant material	Country of origin	Concentration in dried plant material (g/100 g)										
		Curculigine	**5**	Hypoxine	**6**	**1**	**10**	**7**	**3** and **4**	**2**	**8** and **9**	**11**
Hypoxis obtusa complex rhizomes	Zimbabwe Kenya	–	0.33	–	–	2.90	0.17	1.55	0.14	–	0.10	0.06
Hypoxis obtusa rhizomes	Mozambique	–	–	0.12	–	3.71	–	–	0.48	–	–	–
Hypoxis angustifolia rhizomes	Zimbabwe	–	–	–	–	0.07	0.09	1.56	0.01	–	0.12	0.03
Hypoxis angustifolia rhizomes	Madagascar	–	0.08	–	–	–	–	–	–	–	–	
Hypoxis decumbens rhizomes	Brazil	–	0.17	–	–	–	0.17	–	–	–	–	–
Curculigo recurvata rhizomes	Zaire	0.18	0.25	–	–	–	–	–	–	–	–	–
Curculigo recurvata flowers	Zaire	0.04	0.07	–	–	–	–	–	–	–	–	–
Hypoxis interjecta rhizomes	South Africa	–	–	–	0.45	4.37	0.22	0.26	0.19	–	–	–
Hypoxis multiceps rhizomes	South Africa	–	–	–	0.41	3.98	0.18	0.27	0.29	–	–	–
Hypoxis argentea rhizomes	South Africa	–	–	–	0.23	–	–	–	–	–	–	–
Campynema lineare rhizomes	Tasmania	–	–	–	–	0.91	–	0.46	0.32	–	–	–
Hypoxis nyasica rhizomes	Malawi	–	0.16	–	–	5.40	0.15	3.12	0.29	–	0.03	–

jectin, curculigine, hypoxine, rooperol and nyasol were obtained as described. Fig. 1 shows the chemical structures of compounds **1–11**. The purity of these compounds was tested by HPLC.

Preparation of crude extracts

The botanical material was homogenized and extracted with methanol for 24 h. After centrifugation and rinsing with methanol, the solvent was evaporated under vacuum at 40°C. A small aliquot of the residue was dissolved in methanol, passed through a Millipore filter and 2–10 μl of the solution were directly injected into the chromatographic system.

RESULTS AND DISCUSSION

Table I gives the retention times and capacity factors of all the compounds isolated. The results show that the polarity, both as sugar units and as phenolic hydroxy groups, plays an important part in the determination of the retention times.

Fig. 2 gives the chromatograms of a standard solution containing all the tested compounds and of the extract of *Hypoxis obtusa* from Zimbabwe. The peak identification was performed on the basis of the chromatographic retention time and by the simultaneous injection of a standard.

To verify the linearity of the detector response, suitable amounts of the substances were weighed and dissolved in calibrated flasks. Measured volumes of the solutions were injected and chromatographed. The data were plotted to show the relationship between the amount injected and the corresponding peak area. The regression equations and their correlation coefficients are listed in Table II. The minimum limit of detection is 10 μg/g of plant material for hypoxoside, nyasoside, mononyasine A, mononyasine B and nyasol, and 30 μg/g of plant material for nyasicoside, nyaside, obtuside A, obtuside B and rooperol at a signal-to-noise ratio of 3. Repeated analyses of the methanol extract samples spiked with standards at three different concentrations gave within-assay relative standard deviations (R.S.D.) ranging from 3.5 to 8.3%. The between-assay R.S.D. ranged from 4.5 to 9.6%.

Table III gives the analytical results for the plant materials examined.

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