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A Method to Separate Lignoids from *Virola* Leaves

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A simplified technique for the isolation of lignoid mixtures in plant leaves has been developed. The technique provides an economic and rapid means for the determination of the most abundant lignoid skeleton by proton magnetic resonance spectroscopy as well as a method of screening small or large quantities of plant leaves for lignoids. © 1997 by John Wiley & Sons, Ltd.

Keywords: Virola oleifera; V. pavonis; Myristicaceae; lignoid determination; lignan-7-ols; 8,4′-oxyneolignan; screening.

INTRODUCTION

The genus *Virola* (Myristicaceae) comprises approximately 35 species which are distributed in neotropical countries (Rodrigues, 1980). Their geographical and taxonomic distribution has been used in studies of chemical ecology (Paulino Filho, 1985), especially of the Amazon forest. The bark resin, derived from various *Virola* species, provides hallucinogenic snuff and an arrow poison, and the species has thus attracted phytochemical examination (Holmstedt *et al.*, 1980). The genus is well known to be an abundant source of lignans and neolignans, and its important physiological actions have been reported (MacRae and Towers, 1985). A particularly striking finding is the observed high levels of lignoids found in human urine (enterolignans), following dietary intake of vegetable fibre and grain (Bannwart *et al.*, 1989), which have been suggested to have cancer-protective effects (Adlercreutz *et al.*, 1991). Investigations of lignoids in plants were mainly carried out with trunk wood (Fernandes, J. B. *et al.*, 1980), bark, fruit (Lemus and Castro, 1989), fruit pericarp, aril, seed coat and kernel (Kato *et al.*, 1990), while little is known of the distribution of these compounds in the leaves (Martinez *et al.*, 1985).

The apparent lack of phytochemical data may be due in part to the nature of the crude extracts of *Virola* leaves, which generally yield a sticky residue composed of chlorophylls and other colouring matters which are difficult to resolve by chromatographic methods. The general procedure for the removal of colouring matter from plant leaves includes the treatment of green extracts with freshly activated charcoal or by passing through a charcoal column (Nair *et al.*, 1990), as well as by lead acetate adsorption. There are, however, disadvantages to these procedures: metabolite losses can occur through occlusion in the precipitate and a residuum of lead acetate remains in solution and can cause problems in subsequent stages. As part of our continuous research work on the *Virola*, we have examined the lignoid constituents of the leaves of *V. surinamensis* (Rol.) Warb. (Barata *et al.*, 1978; Queiroz Paulo, 1982; Santos and Barata, 1990) of *V. oleifera* (Schott) A. C. Smith (Fernandes, A. M. A. P. *et al.*, 1993, 1994) and of *V. pavonis* (A. DC.) A. C. Smith (Ferri and Barata, 1991, 1992). The crude extracts of the leaves were systematically submitted to chlorophyll elimination yielding purified fractions free of colouring matter and rich in lignoid mixtures. This resulted in the isolation of seventeen lignoids, including nine new compounds. The present work describes the use of this simplified methodology for an efficient separation of the lignans and neolignans from apolar and polar crude extracts of the leaves of *V. oleifera* and *V. pavonis*.

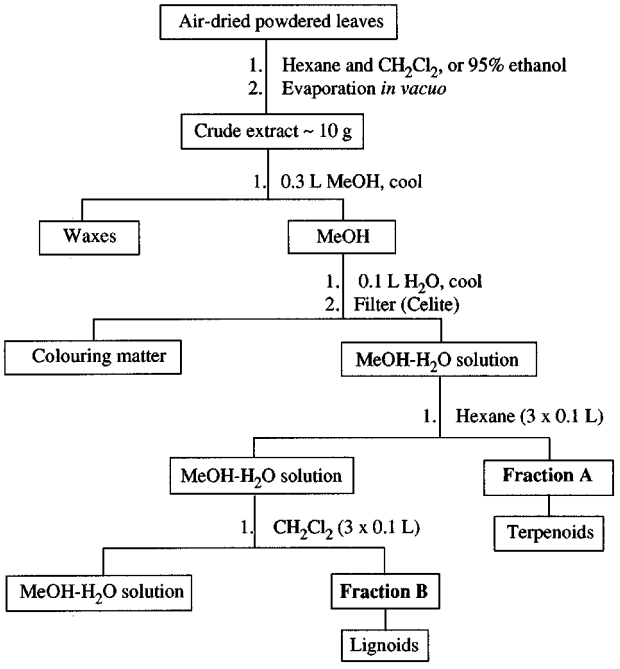

Figure 1. General procedure for the elimination of chlorophyll in extracts of leaves of *Virola*.

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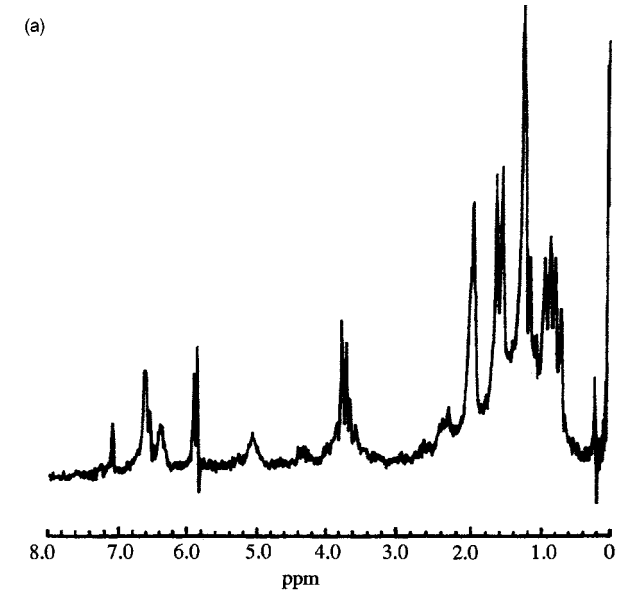


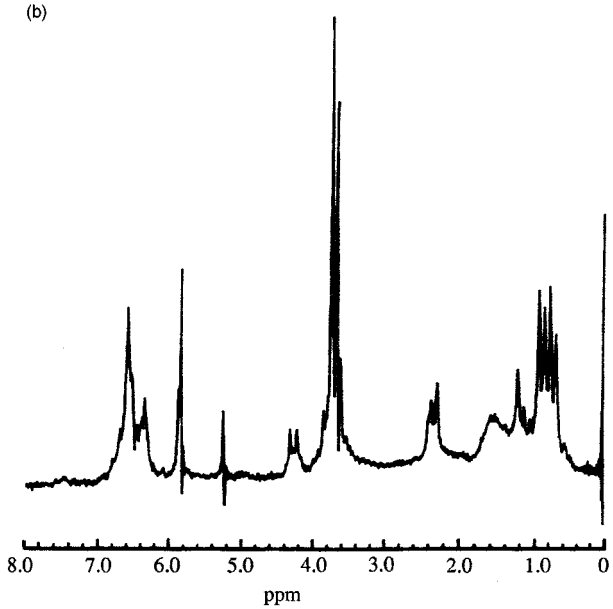
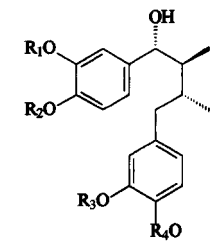
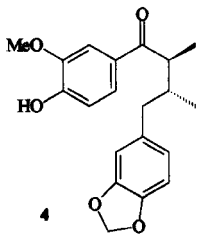
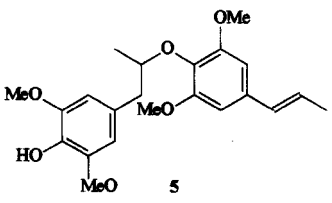


Figure 2. Proton magnetic resonance spectra (80 MHz; carbon tetrachloride; δ) of (a) a crude ethanol extract from the leaves of *V. oleifera*; and (b) a chlorophyll-free fraction (fraction **B**) obtained following chlorophyll elimination from the crude extract above (following the procedure described in the Experimental section).



	R1	R2	R3	R4
1	—CH2—		Me	Me
2	Me	Me	—CH2—	
3	—CH2—		—CH2—	





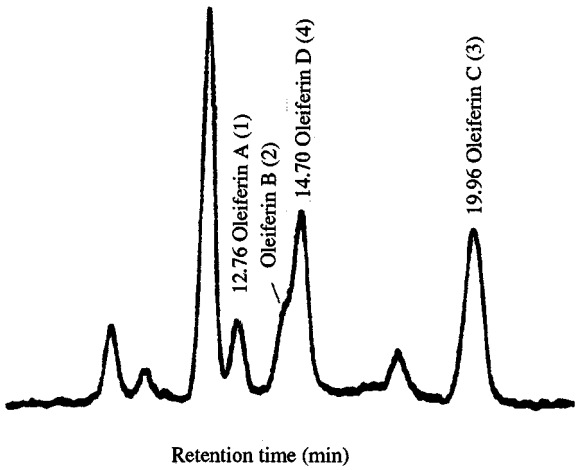


Figure 3. An HPLC chromatogram of a chlorophyll-free fraction (fraction **B**) obtained from *V. oleifera*; (the analytical protocol is described in the Experimental section).

EXPERIMENTAL

General. Proton magnetic resonance (^{1}H NMR) spectra were recorded at 60 and 80 MHz in carbon tetrachloride with tetramethylsilane (TMS) as internal standard. High performance liquid chromatography (HPLC) was performed on a Waters 600E-MDS instrument using a Nova-Pak RP-C18 column (150 × 3.9 mm i.d.; 4 μm particle size; Milford, MA, USA). The mobile phase was methanol:water (7:3) at a flow rate of 1.0 mL/min; detection was by UV detector operating at a wavelength of 280 nm.

Plant material. Leaves of *V. oleifera* were collected by Dr Gentil Godoy (Atlantic Forest Reserve, Ubatuba, SP, Brazil) in May 1990, and authenticated by Dr Jorge Tamashiro (Departamento de Botânica, Unicamp, Campinas, SP, Brazil). A voucher specimen is deposited in the Herbarium of Unicamp. The leaves of *V. pavonis* were collected in the Amazonian forest by Dr Hipólito F. Paulino Filho, near the Guaporé river (Rondônia State, Brazil) and authenticated by Dr William A. Rodriques (Instituto Nacional de Pesquisas da Amazônia-INPA, Manaus, Brazil). A voucher specimen is deposited in the Herbarium of INPA.

Extraction and chlorophyll elimination. In order to eliminate colouring matter, the air-dried powdered leaves were extracted with hexane and dichloromethane successively, or with 95% ethanol at room temperature. The crude extracts were evaporated *in vacuo*. The green gummy extracts were dissolved in methanol, cooled (5°C; 30 min) and filtered to remove the precipitated waxes. The green methanolic filtrates were diluted further with water in order to obtain a 7:3 ratio of the solvents. Depending on the plant, the methanol:water composition should be slightly modified to 3:2 or 4:1. Cooling the cloudy methanol:water solution to 5°C accelerated the flocculation of chlorophyll, thus permitting separation from the methanol:water solution by filtration with methanol-washed Celite.

Separation of lignoid mixtures. The colourless methanol: water solutions were extracted first with hexane and then with dichloromethane. Evaporation of the hexane and dichloromethane solutions at reduced pressure afforded two pale yellow oily fractions, **A** and **B** respectively (Fig. 1).

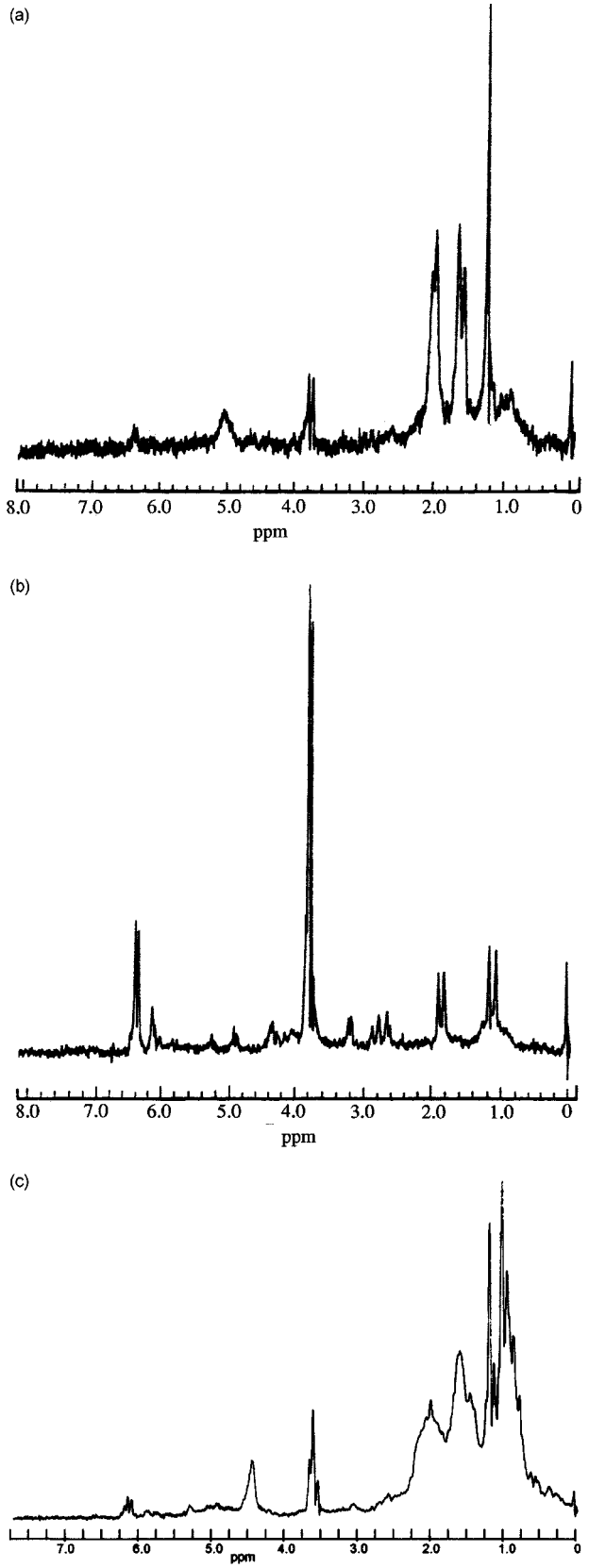


Figure 4. Proton magnetic resonance spectra (60 MHz; carbon tetrachloride; δ) of (a) a crude hexane extract from the leaves of *V. pavonis;* (b) a chlorophyll-free fraction (fraction **B**); and (c) fraction **A**, both obtained following chlorophyll elimination from the crude extract above (following the procedure described in the Experimental section).

RESULTS AND DISCUSSION

The leaves of *V. oleifera* were extracted at room temperature by percolation with hexane (nonpolar extract) followed by dichloromethane (intermediate polarity extract). In a parallel experiment we have tested the efficiency of the extraction procedure using a 95% aqueous ethanol extract (polar extract) since this solvent would combine the extraction capabilities of the two previous solvents.

In order to eliminate chlorophylls and other colouring matter, the deep green leaf extracts were dissolved in methanol, and water was added in order to reach a 7:3 ratio (Fig. 1). The resulting solution should be filtered off over a layer of pre-washed (with methanol) Celite. Hexane and dichloromethane were used to partition the methanol:water solution, yielding yellowish fractions showing the typical absorption of lignoids in the ^{1}H NMR spectra. Our results have shown that the crude 95% aqueous ethanol extract was better than those obtained with hexane and dichloromethane. ^{1}H NMR and thin layer chromatographic (TLC) analysis revealed that the chlorophyll-free fractions **A** and **B** from the 95% ethanol extract were similar to those from the hexane and dichloromethane extracts, but the yields from the former were slightly better than those obtained from the latter. The methodology using 95% ethanol seems to be more attractive due to the use of a less expensive solvent and the reduced time needed for the development of the procedure. Additionally, ^{1}H NMR spectral analysis of the chlorophyll-free fraction **B** (Fig. 2b) from the crude 95% ethanol extract (Fig. 2a) clearly showed characteristic signal patterns belonging to the 1,4-diaryl-2,3-dimethylbutan-1-olic skeleton, methylenedioxy and aromatic methoxyl groups. Upon irradiation at δ1.55, the two methyl doublets and the doublet at δ4.30 collapse into three singlets and the signal δ2.40 became two doublets supporting the presence of the lignan-7-ols, oleiferins A (**1**), B (**2**), C (**3**) and D (**4**). Identification of the respective HPLC peaks shown in Fig. 3 were made by co-injection experiments using authentic lignan-7-ols, the most abundant lignan skeleton from *V. oleifera* leaves (Fernandes, A. M. A. P. *et al.*, 1993).

The chlorophyll elimination procedure above was applied to extracts of *V. pavonis*, a little known species restricted to the Guaporé river region of the Amazon forest (Rodrigues, 1980). The ^{1}H NMR spectrum of the chlorophyll-free fraction **B** (Fig. 4b) from the crude hexane extract (Fig. 4a) showed signals which corresponded to a known β-aryloxy-arylpropane skeleton, the 8.4′-oxyneolignan **5**, the major constituent isolated from the leaves (Ferri and Barata, 1992). On the other hand, fraction **A** afforded a rich source of terpenoid mixtures, as shown in the ^{1}H NMR spectrum of the chlorophyll-free fraction **A** (Fig. 4c).

In summary, the above separation is a useful, economical and rapid procedure for the isolation of lignoid and terpenoid mixtures from plant leaves. As no sorbent is present during the pigment removal, the phenomena of irreversible adsorption and contamination do not cause problems, and this fact is of special importance for the separation of crude extracts. The technique provides a

means to detect the presence and carbon skeleton of small quantities of lignoids and may be readily applied to chemotaxonomic studies, and pharmacological and toxicological screenings, and HPLC analysis of these compounds in leaves.

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