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SEPARATION OF NATURAL POLAR SUBSTANCES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, CENTRIFUGAL THIN-LAYER CHROMATOGRAPHY AND DROPLET COUNTER-CURRENT CHROMATOGRAPHY

FULVIA ORSINI and LUISELLA VEROTTA*
Dipartimento di Chimica Organica e Industriale, Via Venezian 21, 20133 Milan (Italy)

SUMMARY

Reversed-phase high-performance liquid chromatography, centrifugal thin-layer chromatography and droplet counter-current chromatography are complementary, highly efficient techniques for isolating natural, very polar, substances from plant sources. The choice of method can be made directly by a preliminary thin-layer chromatographic investigation. An application to the isolation of some new triterpenic glycosides in *Passiflora quadrangularis* L. is described.

INTRODUCTION

Passiflora extracts have been wisely used in the folk medicine of Europe and America because of their sedative and antihypertensive properties. Many *Passiflora* spp. have already been investigated, and some indole alkaloids of the harman type, C- and O-glycosyl flavonoids, cyanogenic glycosides, and a cyclopropane triterpenic glycoside (passiflorine) have been identified[1–5]. In spite of the different classes of compounds found, the active principles of *Passiflora* spp. have not yet been recognized, and pharmacological tests are currently under investigation. Prior to biological testing, we have studied the applicability of a number of isolation methods to the purification of individual constituents of the aqueous soluble fraction of *Passiflora quadrangularis* L.

EXPERIMENTAL

Gel-filtration column chromatography (GFCC)

Sephadex LH-20 (130 g; Pharmacia) was packed by a slurry technique into a glass column (950 × 30 mm I.D.) and eluted with methanol (300 ml) at a flow-rate of 12 ml/h. A total of 120 2-ml fractions were collected and monitored by thin-layer chromatography (TLC) on silica gel with solvent A.

TLC

Silica gel plates (5 × 10 cm), F_{254} TLC (Merck, Darmstadt, F.R.G.), were

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used. Different eluting systems were applied: (A) chloroform–methanol–*n*-propanol–water (5:6:1:4) (lower layer); (B) ethyl acetate–ethanol–water (8:2:1); (C) chloroform–methanol–*n*-propanol–water (45:60:6:40) (lower layer); (D) chloroform–methanol–water (17:3:2) (lower layer); (E) ethyl acetate–ethanol–water (14:6:3).

For reversed-phase separation, preparative conditions were chosen on reversed-phase thin-layer plates: RP 18 (5 × 10 cm, Merck), eluted with methanol–water (9:1); RP 8 (5 × 10 cm, Merck), eluted with methanol–water (4:1).

Droplet counter-current chromatography (DCCC)

Separations were carried out on a Model-A instrument (Tokyo Rikakikai, Tokyo, Japan) using the solvent systems chloroform–methanol-*n*-propanol–water (5:6:1:4) and chloroform–methanol–*n*-propanol–water (45:60:6:40) in the ascending mode. Fractions of 2 ml were collected at a flow-rate of 0.25 ml/h.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

A Miniprep Jobin-Yvon liquid chromatograph was used. A 40-g quantity of Lichroprep RP-18 (25–40 μm; Merck) was necessary to fill the column (500 × 20 mm I.D.). Two subsequent separations of *ca.* 430 mg of each sample were carried out on the same column. One litre of methanol–water (17:3) was used for each at a flow-rate of 15 ml/min. Fractions of 7 ml were collected and monitored by TLC on silica gel (solvent A) and RP-18 (methanol–water (9:1)).

Centrifugal thin-layer chromatography (CTLC)

Separation was carried out on a Chromatotron Model 7924 T (Harrison Research, Palo Alto, CA, U.S.A.). The starch-bound layer (1 mm) was prepared by mixing 1.2 g of starch (Merck, Art. 1252) with 130 ml of water and 3.0 g of $MgSO_4 \cdot 7H_2O$ (RP; Carlo Erba) and heating the mixture to boiling point. When the solution was clear, silica gel (40 g of PF_{254} + $CaSO_4 \cdot \frac{1}{2}H_2O$) was added in portions, mixed by swirling, then heated for a further 10 min with occasional mixing. The hot mixture was rapidly poured onto the rotor, turning the rotor to avoid unsymmetric loading and tapping the rotor against the cloth-covered surface to release air bubbles. The layer was allowed to dry for at least 48 h at room temperature before drying it in an oven at 70°C for 2 h. After scraping the layer, the rotor was placed on the Chromatotron and repeatedly washed free of inorganic salts with methanol. Complete removal of the two sulphates was controlled with a barium chloride solution. During this operation, some care must be taken to avoid obstruction of the capillary tube. The layer was saturated with the solvents *ca.* 200 ml under our conditions: ethyl acetate–ethanol–water (16:3:2) and then separation could be carried out. The flow-rate was 1.5 ml/min (a faster rate caused too rapid an elution and vitiated resolution). About 20–200 mg of crude products could be applied without loss of resolution. Fractions of 1–2 ml were collected and monitored by TLC on silica gel with solvent B.

RESULTS

A methanolic extract (13.2 g) of *Passiflora quadrangularis* L. leaves was par-

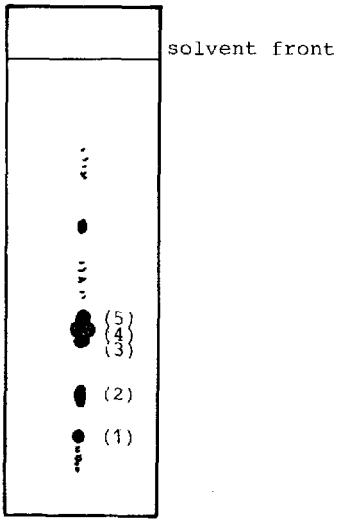
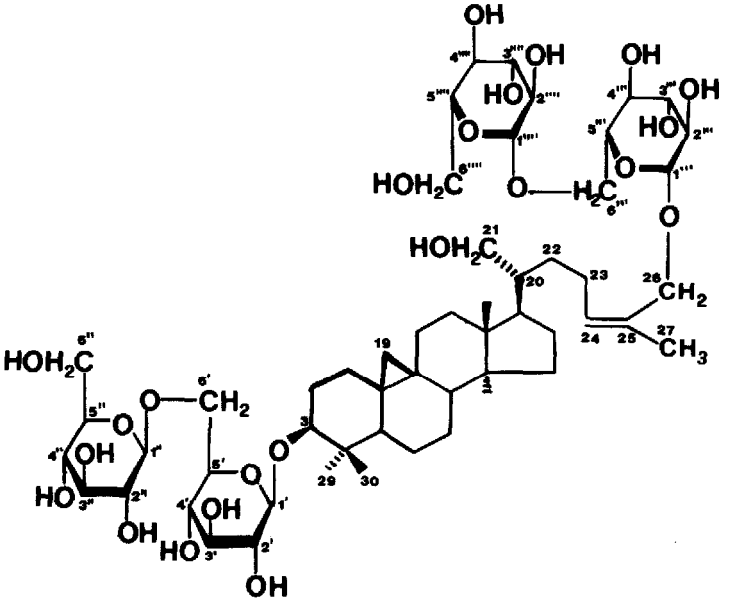


Fig. 1. Thin-layer chromatogram of an aqueous phase of *Passiflora quadrangularis* on Silica gel. Elution system, chloroform–methanol–*n*-propanol–water (5:6:1:4).

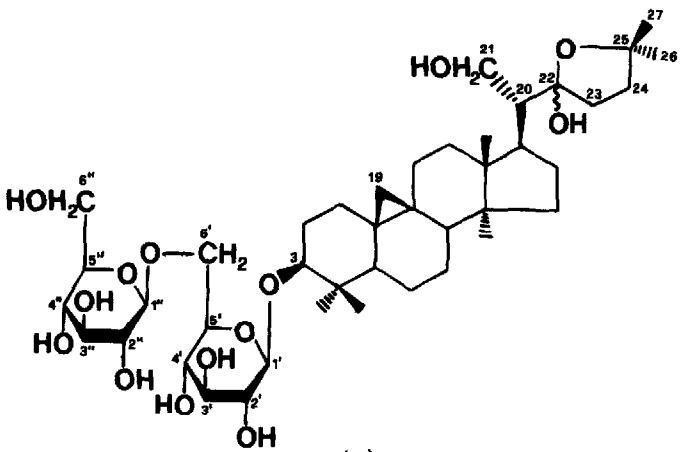
titioned between organic and aqueous phases. The latter (2.60 g) was first investigated by silica gel TLC and showed a series of plum-coloured spots, distributed in the R_F range 0.1–0.4 (see Fig. 1).

A first fractionation (1.590 g) was carried out by elution of a Sephadex LH-20 column with methanol. Three enriched fractions were obtained (79–98 = 566 mg;

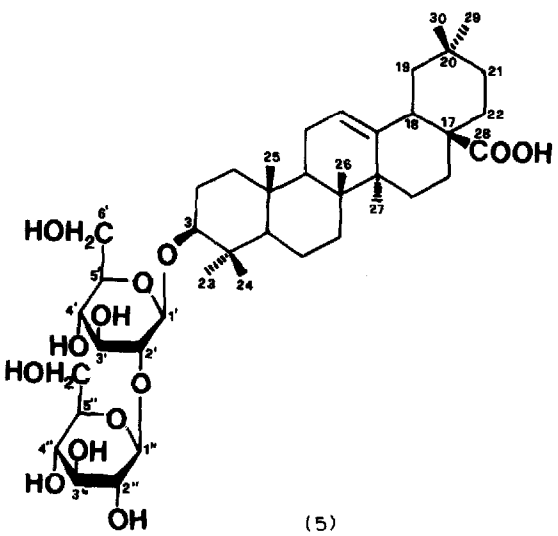


QUADRANGULOSIDE (1)

99–130 = 863 mg; 131–150 = 71 mg). The first one was subjected to DCCC [chloroform–methanol–*n*-propanol–water (5:6:1:4), ascending mode], affording 157 mg of quadranguloside (1) and 58 mg of a mixture (2) (see Fig. 1), which was resolved into three substances by silica gel TLC with different eluting systems (solvents D and E). A preliminary investigation by ^{1}H NMR, ^{13}C NMR and mass spectroscopy indicated that (2) is related to quadranguloside, but further work is in progress to obtain a larger quantity of product.



(4)



(5)

The second fraction was subjected to RP-HPLC on the basis of RP-8 and RP-18 TLC. Using a derivatized silica packing (RP-18, 25–40 μm), 253 mg of compounds (3) + (4) and 400 mg of compounds (5) + (4) were obtained. The latter mixture was subjected to DCCC with chloroform–methanol–*n*-propanol–water (45:60:6:40; ascending mode) and 35 mg of (4) (fractions 155–172) and 150 mg of (5) (fractions 245–273) were isolated.

Repeated CTLC was used to purify 253 mg of (3) + (4) on a starch-bound silica plate, eluted with ethyl acetate–ethanol–water (16:3:2). This allowed us to isolate (3) (fractions 12–17 = 45 mg) and (4) (fractions 7–11 = 130 mg) in pure form.

The structures of the new compounds (1) and (4) isolated were elucidated and compound (5) was identified, both on the basis of acid and enzymatic hydrolysis and on spectral evidence (^{1}H NMR, ^{13}C NMR and mass spectroscopy) of both hydrolysed and intact glycosides. They have been characterized as 9,19-cyclolanost-24*Z*-en-3β,21,26-triol,3,26-di-O-gentiobioside [quadranguloside (1)] and 9,19-cyclolanosta-22,25-epoxy-3β-OH,21,22-triol,-3-β-O-gentiobioside [22-hydroxy-isoquadranguloside (4)]. Compound (5) was identified as oleanolic acid 3-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside. It is the first time that (5) has been isolated from a natural source; it was only previously obtained by hydrolysis of natural precursors[6–8].

On submission of this paper, the structure of compound (3) had not been fully ascertained; it will be published elsewhere, together with details on the structure determination of (4) and (5). The structure elucidation of (1) was the subject of a previous paper[9].

Further work is in progress to resolve the minor components.

DISCUSSION

DCCC is a powerful technique for separating natural, very polar, substances. A quick method for selecting a suitable system consists in subjecting the sample to TLC with the organic layer as eluent[10,11]. Sometimes this technique needs to be supplemented by other methods to improve the separation of complex mixtures.

It has been shown[12–29] that a direct extrapolation of the analytical conditions

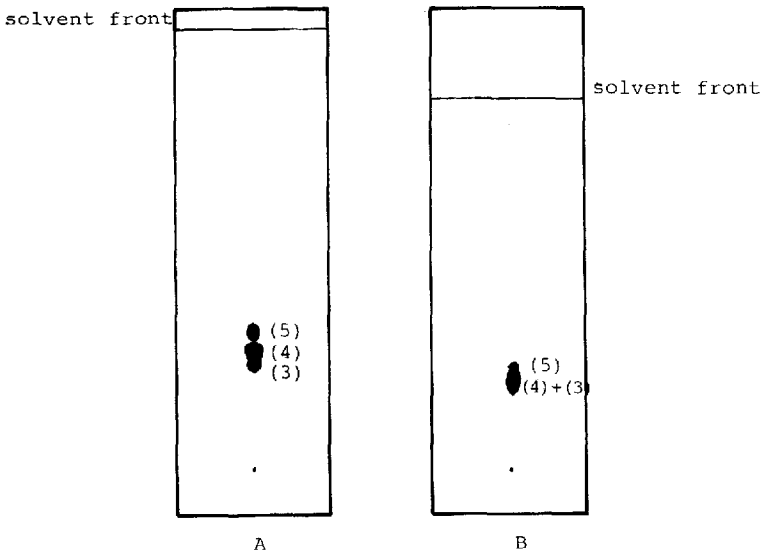


Fig. 2. (A) Separation of fractions 99–130 by RP-TLC on a RP-18 plate, eluted with methanol–water (9:1). (B) Separation of the same sample on a RP-8 plate, eluted with methanol–water (4:1).

in TLC on silica gel or RP layers to a preparative HPLC is possible, since identical or very similar retention mechanisms are responsible for the separation of the respective substances in both chromatographic methods. However, an essential precondition for this transferability is that the adsorbents used in both methods must have identical or at least very similar physical and chemical properties. An empirical rule that we follow to save time is to aim for a maximum R_F of 0.3 in TLC, even if complete separation between the spots is not obtained (see Fig. 2). The separation obtained with an RP-18 packing shows that the highly hydrophobic RP-18 packing requires a slight decrease in solvent strength, in order to give complete separation of (3), (4) and (5).

The great advantages of CTLC have been demonstrated[30–35]; the technique combines good resolving power with low consumption of solvent (generally < 150 ml) and relatively short separation times (30–40 min). A few words on the preparation of a layer suitable for water-containing eluents are in order. The manufacturer recommends the use of a HF silica-gel packing and corn starch, with the addition of magnesium sulphate so as to inhibit cracking and to soften the layer. Our experience in the preparation of the layer led us to the conclusion that a better layer could be obtained by using a silica gel type PF + $CaSO_4 \cdot \frac{1}{2}H_2O$ with the addition of corn starch and magnesium sulphate. After drying (for least 48 h at room temperature, followed by 2 h at 70–80°C), the plate must be washed repeatedly with methanol to remove the inorganic salts.

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