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Note

Separation of natural product sweetening agents using overpressured layer chromatography[a]

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Overpressured layer chromatography (OPLC) is a modified planar chromatographic technique, in which the vapor phase is eliminated by covering the sorbent layer with an elastic membrane under variable external pressure, and the mobile phase is forced through the sorbent layer with a pump[2,3]. The method is particularly advantageous over thin-layer chromatography (TLC), a capillary-controlled system, in that better resolution and more rapid separation can be obtained[2,3].

OPLC methodology that is applicable to both the analytical and on-line preparative separation of natural products has previously been described[4–7]. In this communication, we wish to report the utilization of OPLC for the separation of a number of potently sweet naturally occurring compounds. Analytical separations have been performed on the eight sweet *ent*-kaurene glycoside constituents of *Stevia rebaudiana* (Bertoni) Bertoni leaves, namely, dulcoside A, rebaudiosides A–E, stevioside and steviolbioside. *S. rebaudiana* extracts and stevioside are used as approved sweetening agents in Japan[8], and great interest is currently being shown in rebaudioside A, a major constituent of the plant that has improved properties as a sweetening agent when compared with stevioside[9]. Separations have also been carried out on some highly sweet compounds recently discovered in this laboratory. Polypodoside A is a steroidal saponin constituent of the rhizomes of *Polypodium glycyrrhiza* D.C. Eaton, that was estimated to possess about 600 times the sweetness potency of sucrose[10]. An analytical OPLC system is described for the separation of polypodoside A from its analogues, polypodosides B and C[1], and a preparative OPLC procedure has been applied to purify polypodoside A when present in a crude *P. glycyrrhiza* column chromatographic fraction. Hernandulcin is an intensely sweet sesquiterpene, about 1000 times sweeter than sucrose, that was initially isolated from the leaves of *Lippia dulcis* Trev., and then synthesized from two commercially available ketones by directed aldol condensation[11,12]. An OPLC method is described for the

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[a] Part 17 in the series *Potential Sweetening Agents of Plant Origin*. For part 16 see ref. 1.
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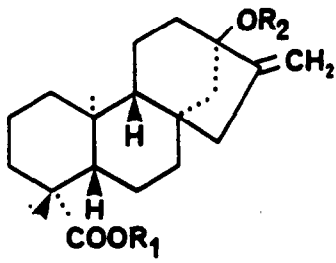
rapid preparative purification of this non-polar compound from an impure synthetic mixture.

EXPERIMENTAL

General procedures

All separations were performed on a Chrompres 25 OPLC instrument (Labor MIM, Budapest, Hungary). The eluting solvent was delivered at a suitable flow-rate with an LDC/Milton Roy (Riviera Beach, FL, U.S.A.) mini-pump VS, and the cushion pressure was kept at 15–17 bar during all separations. Plate-edge impregna-



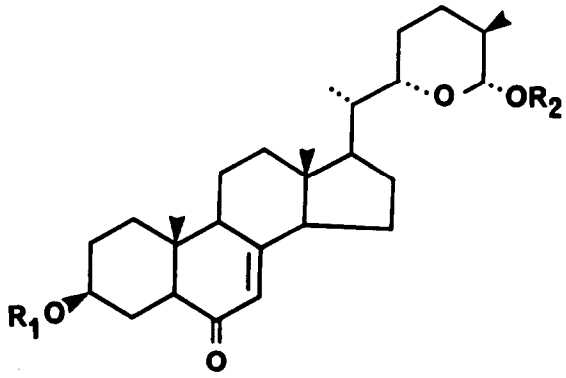
	R_1	R_2
1	β-glc^2-β-glc	β-glc^2-β-glc |^3 β-glc
2	β-glc^2-β-glc	β-glc^2-β-glc
3	β-glc	β-glc^2-β-glc |^3 β-glc
4	β-glc	β-glc^2-α-rha |^3 β-glc
5	β-glc	β-glc^2-glc
6	H	β-glc^2-β-glc |^3 β-glc
7	β-glc	β-glc^2-α-rha
8	H	β-glc^2-β-glc

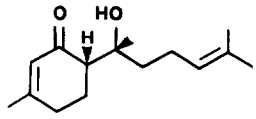
Glc = D-glucopyranosyl; rha = L-rhamnopyranosyl

tion was effected using Impres II polymer suspension (Labor MIM). All solvents used were analytical grade.

Analytical separations were carried out on precoated TLC and high-performance TLC (HPTLC) silica gel 60 F_{254} aluminium- and glass-backed plates, respectively, 10 × 20 cm, 0.2 mm layer thickness (Merck, Darmstadt, F.R.G.). Solute mixtures used in these separations were constituted in a final concentration of about 1 mg/ml with equal quantities of each plant isolate present. Each mixture was applied to the sorbent layers in 5–30-μg quantities. Prior to each separation, the plate was preconditioned with hexane at 2 ml/min for 12 min, to displace air in the adsorbent layers. A flow-rate of 0.2 ml/min was used for all analytical OPLC separations. Chromatograms were evaluated using a Zeiss KM 3 chromatogram spectrophotometer (Zeiss, Oberkochen, F.R.G.).

Preparative separations were made on precoated PSC-silica gel 60 F_{254} plates, 20 × 20 cm, 1 mm layer thickness, with a 4 × 20 cm preconcentration zone (Merck). Such separations were conducted using a previously described on-plate injection technique[7], in which sample injection was accomplished using a Multifit B-D syringe, and, prior to injection, the plates were equilibrated with each solvent system to be used for about 30 min at a flow-rate of 2 ml/min. Eluted fractions were collected in a Buchler (Fort Lee, NJ, U.S.A.) Fractomette Alpha-200 automatic fraction collector. The purity of these fractions was checked by TLC using silica gel 60 PF_{254} aluminium backed plates (Merck).





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	R_1	R_2
9	β-glc²-α-rha	α-rha
10	β-glc	α-rha
11	β-glc	α-rha-3-CH_3

Glc = D-Glucopyranosyl; rha = L-rhamnopyranosyl

Samples

The sweet *ent*-kaurene glycosides, dulcoside A (**7**), rebaudiosides A (**3**), C (**4**), D (**1**), E (**2**) and stevioside (**5**), were isolated in pure form from *S. rebaudiana* leaves as previously described[13,14]. Rebaudioside B (**6**) and steviolbioside (**8**) were prepared from rebaudioside A and stevioside, respectively, by alkaline hydrolysis[13]. Pure polypodosides A (**9**), B (**10**) and C (**11**) were isolated from the rhizomes of *P. glycyrrhiza* collected in Oregon[1,10]. A crude sample of polypodoside A was also obtained from this plant source. (±)-Hernandulcin (**12**) was synthesized from 3-methyl-2-cyclohexen-1-one and 6-methyl-5-hepten-2-one, as published previously[11,12], and was used in this investigation as a highly impure synthetic reaction product.

RESULTS AND DISCUSSION

Separation of the sweet glycosides of Stevia rebaudiana

Thus far, it has not proven possible to resolve all eight sweet diterpene glycosides from *S. rebaudiana* using TLC, and when a published solvent system[14] was employed for this separation, only partial resolution resulted for rebaudiosides D (**1**) and E (**2**), rebaudioside C (**4**) and stevioside (**5**), and rebaudioside B (**6**) and dulcoside A (**7**) (Fig. 1a). When OPLC was applied on normal TLC plates using the same solvent system (Fig. 1b), the development time was reduced by a factor of eight, although no substantial improvement of resolution was apparent between these three pairs of glycosides. However, compounds **4** and **5** were resolved with baseline separation, and compounds **6** and **7** substantially resolved by OPLC in this same solvent system using HPTLC plates (Fig. 1c). Although rebaudiosides D (**1**) and E (**2**) were not separated using this OPLC procedure, it may be pointed out that these two compounds are somewhat more polar than the other *S. rebaudiana* sweet glycosides in being preferentially water soluble, whereas the other six compounds (**3–8**) are extractable into 1-butanol from aqueous solutions[13,14].

*Separations of polypodosides A–C (**9–11**)*

Polypodosides A–C (**9–11**) are polar steroidal saponins that occur in the rhizomes of the North American fern, *P. glycyrrhiza*. Unlike the intensely sweet compound, polypodoside A (**9**), polypodoside B (**10**) is only slightly sweet, while polypodoside C (**11**) is completely devoid of this effect[1,10]. Although a mixture of these compounds was separated easily by TLC on silica gel, this analytical separation took 3 h to complete (Fig. 2a). OPLC on normal TLC plates, using a slightly modified solvent system, enabled the separation of these compounds in only 15 min (Fig. 2b).

An impure sample of polypodoside A (**9**) was obtained after the gravity column chromatographic separation of a 1-butanol extract of *P. glycyrrhiza* rhizomes, by elution with chloroform–methanol (6:1, v/v)[10]. This sample was free of polypodosides B and C, but highly contaminated with other unknown polar plant constituents. The same solvent system as shown in Fig. 2b was chosen for OPLC, although a higher flow-rate of 1.0 ml/min was used. A 55-mg sample of the impure polypodoside was dissolved in 1 ml of mobile phase, filtered, and applied to the preconditioned plate by on-line injection[7]. Altogether 12 10-ml fractions were collected, and 16 mg of pure polypodoside A (**9**) were obtained in fractions 9–11. The total time for this separation was about 2 h.

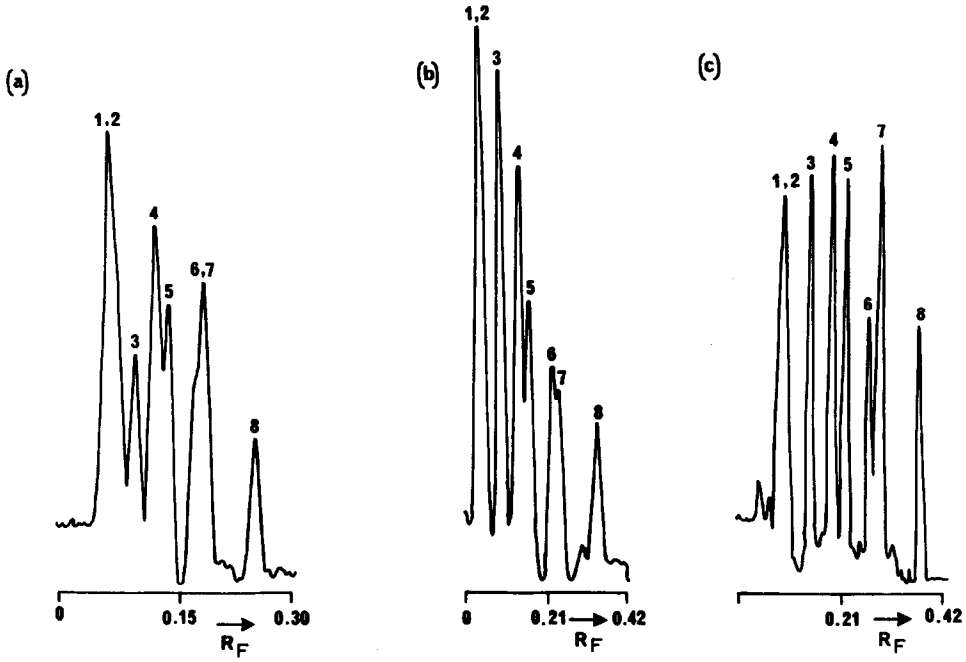

Fig. 1. Analytical separation of sweet *ent*-kaurene glycosides isolated from *S. rebaudiana*. Peaks: 1 = rebaudioside D; 2 = rebaudioside E; 3 = rebaudioside A; 4 = rebaudioside C; 5 = stevioside; 6 = rebaudioside B; 7 = dulcoside A; 8 = steviolbioside. (a) Normal TLC; mobile phase, ethyl acetate–ethanol–water (130:27:20, v/v/v); UV detection (after spraying plate with 60% (w/v) sulfuric acid, and heating for 10 min at 110°C), 325 nm; development time, 135 min; (b) OPLC with TLC plate: solvent and UV detection as for (a); flow-rate, 0.2 ml/min; development time, 16 min; (c) OPLC with HPTLC plate: solvent, UV detection, flow-rate, and development time as for (b).

*Purification of synthetic hernandulcin (***12***)*

When synthetic (±)-hernandulcin (**12**) was prepared by directed aldol condensation from 3-methyl-2-cyclohexen-1-one and 6-methyl-5-hepten-2-one, nearly a 50% yield occurred in this reaction[11,12]. However, in addition to unreacted starting materials, three minor reaction products have also been indentified[12], which must also be removed from the final product. In the present investigation, the work-up of **12** from a mixture of synthetic precursors and reaction products that had been stored for several months was accomplished by OPLC on normal TLC plates, using hexane–ethyl acetate (10:3, v/v) as mobile phase, at a flow-rate of 1 ml/min. The sample (100 mg) was dissolved in 1 ml of mobile phase, and injected onto the preequilibrated sorbent layer. Altogether nine fractions were collected, each of 8 ml, with pure henandulcin (6 mg) collected into fractions 6 and 7. The time of separation was 72 min.

CONCLUSIONS

It has been shown in this investigation that OPLC is effective in both the analytical and preparative separation of three structural classes of high-intensity

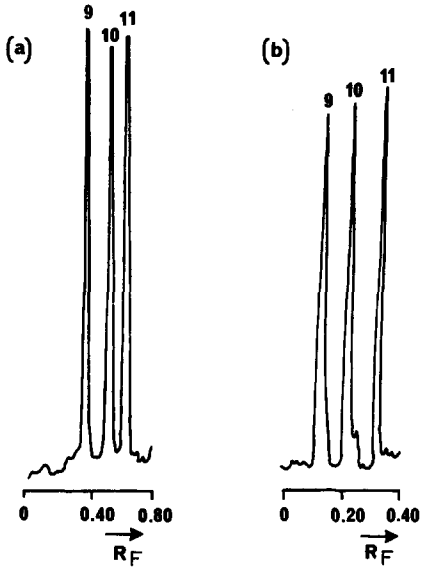

Fig. 2. Analytical separation of sweet steroidal saponins isolated from *P. glycyrrhiza*. Peaks: 9 = polypodoside A; 10 = polypodoside B; 11 = polypodoside C. (a) Normal TLC: mobile phase, chloroform–methanol–water (6:3:1, v/v/v; lower layer); UV detection, 254 nm; development time, 180 min. (b) OPLC with TLC plate: solvent, hexane–chloroform–methanol–water (0.8:6:3:1, v/v/v; lower layer); UV detection, as for (a); flow-rate, 0.2 ml/min; development time, 15 min.

natural sweeteners, which ranged from highly polar *ent*-kaurene glycosides (**1–8**) and steroidal saponins (**9–11**), to a non-polar sesquiterpene (**12**). In analytical separations of mixtures of the first two categories of these substances, the separation speed achieved was 8–12 times faster by OPLC than by conventional TLC. Using an on-line injection technique that avoided the necessity to streak plates with solutes, pure samples of polypodoside A (**9**) and (±)-hernandulcin (**12**) were obtained by preparative OPLC from highly impure starting materials in a period of 2 h or less. In both cases, the use of this preparative procedure avoided the necessity for additional protracted conventional purification techniques. As has been noted previously[4], the greatest utility of OPLC for the separation of bioactive natural products would seem to be for the purification of small (50–100 mg) amounts of partially purified samples.

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