Journal of Chromatography, 362 (1986) 375–381
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 695

CHROMATOGRAPHY OF TANNINS

II*. PREPARATIVE FRACTIONATION OF HYDROLYZABLE TANNINS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

TAKUO OKUDA*, TAKASHI YOSHIDA, TSUTOMU HATANO, KAZUFUMI YAZAKI, REIKO KIRA and YOSHITAKA IKEDA
Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700 (Japan)
(Received April 1st, 1986)

SUMMARY

Centrifugal partition chromatography with an apparatus which employs rotary joints has been applied to the separation of tannins in extracts from several plant species. The results were compared with those obtainable by droplet counter-current chromatography. Combined applications of normal- and reversed-phase developments, and of centrifugal partition chromatography and other chromatographic methods, were also carried out. The results show that centrifugal partition chromatography shortens the development time to a quarter of that with droplet counter-current chromatography, and enables separation of some tannins whose isolation by droplet counter-current chromatography is impracticable. It is also demonstrated that centrifugal partition chromatography is a suitable method, particularly on the preparative scale, for the separation of tannins.

INTRODUCTION

The progress in the development of chromatographic and spectrometric techniques has aided the chemical investigation of tannins, resulting in the isolation of a large number of tannins and the discovery of various biological activities[1]. Among these techniques are droplet counter-current chromatography[2,3] and other new counter-current chromatographic techniques[4]. They do not require a solid support which usually results in losses of tannin by adsorption on various substances, and therefore has advantages over other methods of chromatography, particularly on the preparative scale.

Droplet counter-current chromatography[5] has the advantage over the classical apparatus[6] in an high partition efficiency obtained by simple operation of a simple instrument, and the separation of hydrolyzable tannins has been facilitated by combination of this technique with column chromatography on gels such as Toyopearl

* For Part I, see T. Okuda, K. Mori, K. Seno and T. Hatano, *J. Chromatogr.*, 171 (1979) 313.

0021-9673/86/$03.50 © 1986 Elsevier Science Publishers B.V.

HW-40 and Sephadex LH-20[2,7]. However, it usually takes a few days or longer for fractionation of tannins in a mixture extracted by an organic solvent from the crude extract of a plant species, and this may result in diffusion and hydrolysis of the tannins on the column.

Having used droplet counter-current chromatography for separation of tannins for several years, we have now applied centrifugal partition chromatography with rotary seal joints, which enables the solvent to be pumped fairly quickly into the rotating separation columns in order to shorten the separation time[8].

EXPERIMENTAL

Apparatus

A centrifugal partition chromatograph Model L-90 (Sanki Engineering, Nagaoka-kyo, Kyoto, Japan), comprising a centrifuge in which there are twelve column cartridges each containing a polytrifluoroethylene resin block (150 mm × 40 mm × 40 mm)[8], was used. Each resin block has 50 holes as the separation columns (40 mm × 3 mm) which are connected to each other by fine resin tubes. The internal volume of each cartridge is *ca.* 15 ml. The sample solution and the solvents were pumped into the columns rotating at 1000 rpm, with a pump, Model CPC-LBP-II (Sanki). An UV absorbance monitor, Model CPC-UVM-I, equipped with a cell of light path 0.2 mm (Sanki) was used at 254 nm, and fractions were collected with a fraction collector, Model SF-160K (Toyo Kagaku Sangyo, Tokyo, Japan).

For the fractionation experiment using droplet counter-current chromatography, the apparatus consisted of a hundred Pyrex tubes (1200 mm × 3.2 mm), mutually connected by fine resin tubes. The internal diameter of the glass tubes is the smallest which enables the formation of droplets of the solvent system of which *n*-butanol is the main component of the upper layer.

Solvents

The solvent systems for centrifugal partition chromatography were the same as those for droplet counter-current chromatography: A, *n*-butanol–*n*-propanol–water (4:1:5, v/v/v); B, *n*-butanol–*n*-propanol–water (2:1:3). The lower layer was used as the stationary phase for the normal-phase development, and was pumped into the columns prior to the loading of the sample solution. The upper layer was used as the stationary phase for the reversed-phase development. The selection of the solvent system was made by thin-layer chromatography (TLC) on cellulose plates.

Preparation and fractionation of extracts

The preparation of plant extracts and the fractionation of tannins were carried out as follows.

(i) *Lythrum anceps Makino.* Fresh leaves (1.4 kg) were homogenized in acetone–water (1:1, v/v), and the filtrate was concentrated *in vacuo* at $<40°C$. After extracting with diethyl ether, the mother-liquor was extracted with ethyl acetate. The mother-liquor was evaporated *in vacuo* and the residue was extracted with methanol. A portion (4.3 g) of the residue (77 g) obtained upon evaporation of the methanol solvent was dissolved in the lower layer of the solvent system A (10 ml), and then

pumped into the columns initially at a rate of 0.2 ml/min; 1 h later this rate was increased to 1.0 ml/min and 10-g portions of the eluent were collected.

The tannins in another portion of the residue (3 g) obtained from the methanol solution were fractionated by droplet counter-current chromatography with solvent system A initially at 0.2 ml/min and 1 h later at 0.8 ml/min; 10-g fractions were collected (Fig. 1).

(ii) *Stachyurus praecox Sieb. et Zucc.* The extraction of fresh leaves (2.5 kg) was carried out analogously to that for *Lythrum anceps*, and a portion (3 g) of the ethyl acetate extract (46 g) was subjected to centrifugal partition chromatography using solvent system B for the normal-phase development. The flow-rate was changed in the same way as for the separation of the extract of *Lythrum anceps*.

(iii) *Liquidambar formosana Hance.* The ethyl acetate extract (8.7 g) was obtained from fresh leaves (2.9 kg) analogously to that described for *Lythrum anceps* and a portion (3 g) of the extract was fractionated by centrifugal partition chromatography, using solvent system A, at first by the reversed-phase development, at the same rate as in (i). The residue (0.7 g) obtained upon evaporation of the combined fractions of group I (Fig. 2) (5 g in total) was subjected to normal-phase development using the same solvent system, yielding two groups of fractions, 18–28 and 29–42.

(iv) *Geranium thunbergii Sieb. et Zucc.* The ethyl acetate extract (8.5 g) was obtained from the dried leaves (70 g), and geraniin was crystallized out of an aqueous methanol solution of the extract. The mother-liquor was evaporated, and 2 g of the residue (4.4 g) were fractionated by centrifugal partition chromatography using normal-phase development with solvent system A; and 5-g portions of the eluent were collected.

Analyses of fractions

The residues obtained upon evaporation of the solvent were analyzed by high-performance liquid chromatography (HPLC) with UV detection at 280 nm, and also by diode-array UV detection. Apparatus: LC-6A system (Shimadzu, Kyoto, Japan) equipped with a YMC A312 (ODS) column (150 mm × 6 mm; Yamamura-kagaku, Kyoto, Japan), an UV monitor, SPD-6A (Shimadzu) and a diode-array detector, MCPD-350PC, System II (Union-Giken, Hirakata, Osaka, Japan). The column was kept at 40°C in an oven, and was eluted with 0.05 *M* H_3PO_4–0.05 *M* KH_2PO_4–methanol (2:2:1, v/v/v). The purity of isolated components was also examined by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy using Hitachi R22-FTS (90 MHz for ^1H and 22.6 MHz for ^{13}C) and JEOL FX-200 (200 MHz for ^1H and 50 MHz for ^{13}C) instruments.

Gel-column chromatography

The combined fractions from centrifugal partition chromatography were further separated by column chromatography on Sephadex LH-20 [ethanol-to-methanol gradient elution, or elution with ethanol-water (7:3, v/v)], and on Toyopearl HW-40F [elution with methanol–water (1:1) or ethanol–water (7:3)].

RESULTS AND DISCUSSION

As shown in Fig. 1, the separation of tannins by centrifugal partition chro-

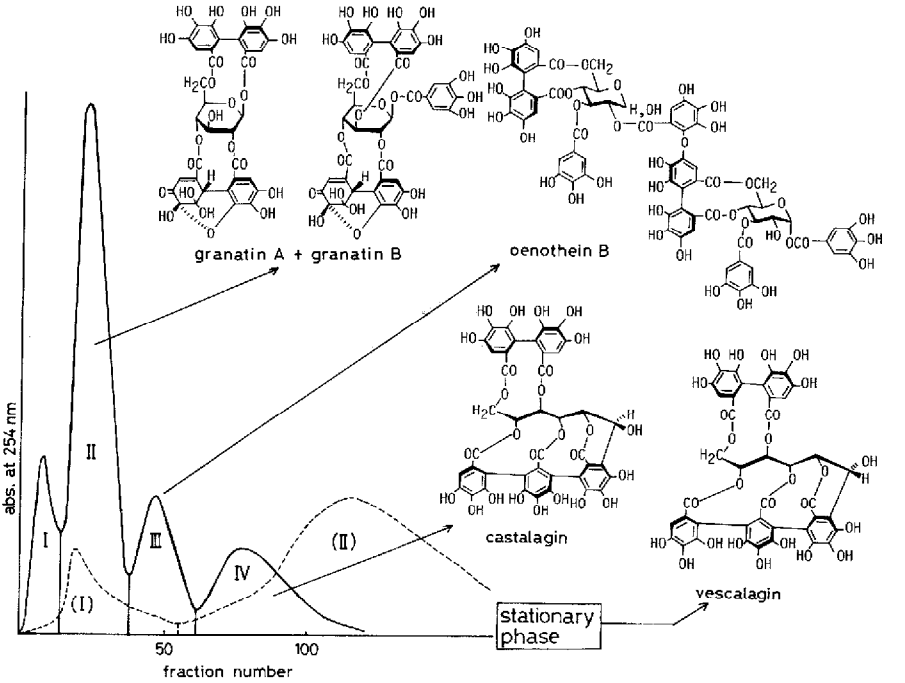

Fig. 1. Centrifugal partition (—) and droplet counter-current (---) chromatograms of tannins in a methanol extract (see text) from *Lythrum anceps* leaves.

matography to an extent analogous to that obtainable by droplet counter-current chromatography was achieved within a quarter of the time required for the latter. Fractions in groups III and IV (Fig. 1) of the ethyl acetate extract from *Lythrum anceps* contain a dimeric hydrolyzable tannin (oenothein B[9], 38 mg) and a *C*-glucosidic tannin (castalagin[10], 85 mg), respectively, which poorly transfer to the mobile phase, and another *C*-glucosidic hydrolyzable tannnin (vescalagin[11], 51 mg) is present in the stationary phase. Although granatin A[12] and granatin B[13] have been separated by droplet counter-current chromatography within a reasonable time[13], collection of the components of groups III and IV by droplet counter-current chromatography is almost impracticable since even collection of the components of group II required more than 60 h.

The ethyl acetate extract from *Stachyurus praecox* leaves, obtained in an analogous way, was separated into three groups of compounds: group I (fractions 1–17, 0.6 g, praecoxins C and E[14]); group II (fractions 18–41, 1 g, praecoxins A, B, D, casuarictin, tellimagrandin I, 1,2,6-tri-*O*-galloyl-*β*-D-glucose, rugosin C[14], guavin A[9] and rugosin F, which is a dimeric tannin[14]); group III (fractions 42–70, 1.4 g, strictinin, pedunculagin and casuarinin, which is a *C*-glucosidic tannin[14]). Among these tannins, of interest is the separation of praecoxin C from rugosin C (Fig. 3), as praecoxin C has a labile depside group and can be transformed into rugosin C under mild conditions[14].

The tannins in the ethyl acetate extract from *Liquidambar formosana* leaves have been fractionated into three groups by centrifugal partition chromatography

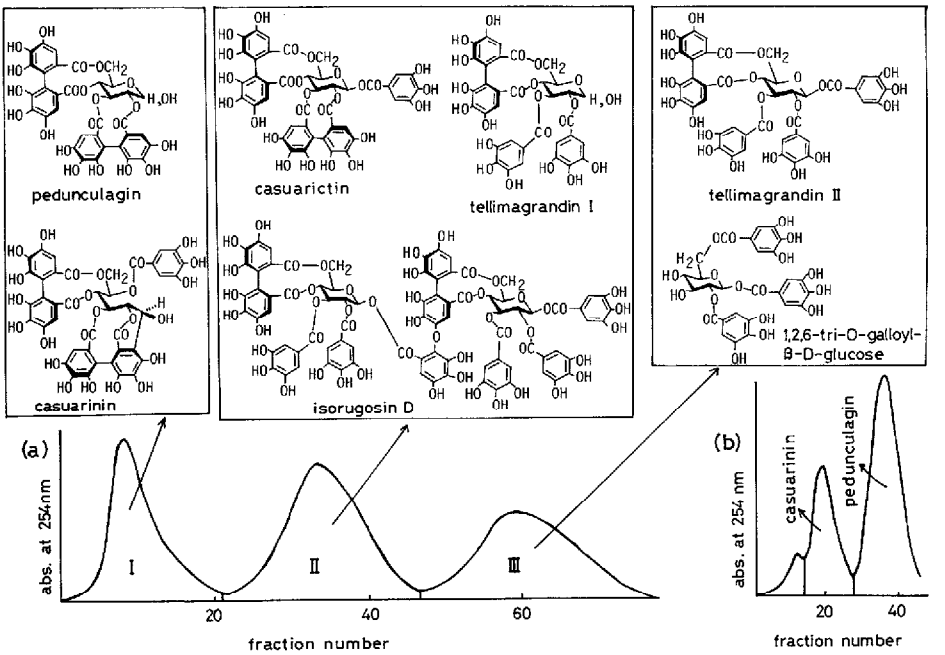

Fig. 2. Centrifugal partition chromatograms of tannins in an ethyl acetate extract from *Liquidambar formosana* leaves. Fractions in group I of the chromatogram (a) (normal-phase development) were subjected to reversed-phase development (b).

using reversed-phase development for about 25 h (Fig. 2). As expected from the results described above, the *C*-glucosidic tannin (casuarinin) was in group I eluted first, and the dimeric hydrolyzable tannin (isorugosin D[15]) was in the second group. Although each group of fractions contained two or three components, this result shows that two components of similar structures, *i.e.*, pedunculagin and tellimagrandin I, and also casuarictin and tellimagrandin II[16], can be separated from each other in a single development. Normal-phase centrifugal partition chromatography of group I induced the separation of two components, casuarinin (77 mg) and pedunculagin (0.4 g) as shown in Fig. 2.

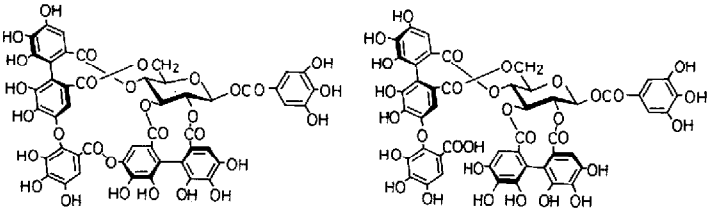

Fig. 3. Structures of praecoxin C and rugosin C.

Elaeocarpusin (= ascorgeraniin)[17,18], a minor component in *Geranium thunbergii*, is an ascorbic acid–tannin condensate and is very soluble in water. It decomposes to geraniin and corilagin when kept in an aqueous solution for several hours. This labile polar compound (38 mg) was isolated effectively from the other components of the plant extract by centrifugal partition chromatography (Fig. 4).

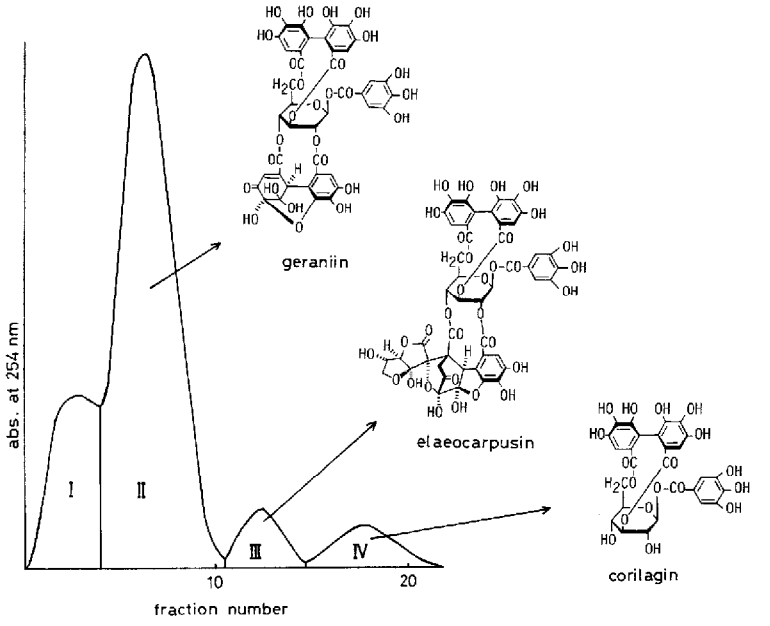

Fig. 4. Centrifugal partition chromatograms of a tannin mixture (see text) extracted from *Geranium thunbergii* leaves.

CONCLUSIONS

Because of the properties of tannins which are labile in solution and easily combine with other substances, centrifugal partition chromatography which does not require a solid support and can separate polyphenolic compounds in a relatively short time without much decomposition and diffusion of each component has advantages over other chromatographic methods. The use of normal- and reversed-phase developments, together with other chromatographic methods, often gives the best results for the isolation of tannins.

In spite of the progress in the development of instrumentation used for determination of the structures of organic compounds, the large molecules of tannins, most of which do not crystallize, require comparatively large amounts of sample for their structure determination. The study of the various biological activities of tannins and related compounds[1] also requires large amounts of samples. The application of centrifugal partition chromatography should meet these requirements.

REFERENCES

1 T. Okuda, *Fragrance J.*, 66 (1984) 34.
2 T. Yoshida, T. Okuda, M. U. Memon and T. Shingu, *J. Chem. Soc., Perkin Trans. 1*, (1985) 315.
3 T. Yoshida, Y. Maruyama, M. U. Memon, T. Shingu and T. Okuda, *Phytochemistry*, 24 (1985) 315.
4 L. J. Putman and L. G. Butler, *J. Chromatogr.*, 318 (1985) 85.
5 T. Tanimura, J. J. Pisano, Y. Ito and R. L. Bowman, *Science (Washington, D.C.)*, 169 (1970) 54.
6 L. C. Craig and D. Craig, in A. Weisberger (Editor), *Technique of Organic Chemistry*, Interscience, New York, 2nd ed., 1956, p. 3.
7 T. Okuda, T. Hatano and N. Ogawa, *Chem. Pharm. Bull.*, 32 (1984) 4662.
8 W. Murayama, T. Kobayashi, Y. Kosuge, H. Yano, Y. Nunogaki and K. Nunogaki, *J. Chromatogr.*, 239 (1982) 643.
9 T. Yoshida, T. Hatano, M. Matsuda, T. Okuda and T. Shingu, *27th Symposium on the Chemistry of Natural Products, Hiroshima, 1985*, Hiroshima University, Hiroshima, 1985, Symposium papers, p. 718.
10 W. Mayer, H. Seitz and J. C. Jochims, *Liebigs Ann. Chem.*, 721 (1969) 186.
11 W. Mayer, H. Seitz, J. C. Jochims, K. Schauerte and G. Schilling, *Liebigs Ann. Chem.*, 751 (1971) 60.
12 T. Okuda, T. Yoshida, K. Mori and T. Hatano, *Heterocycles*, 15 (1981) 1323.
13 T. Okuda, T. Hatano, H. Nitta and R. Fujii, *Tetrahedron Let.*, 21 (1980) 4361.
14 T. Okuda, T. Hatano and K. Yazaki, *Chem. Pharm. Bull.*, 31 (1983) 333.
15 T. Okuda, T. Hatano, R. Kira, T. Kaneda and M. Yoshizaki, *32nd Annual Meeting of the Japanese Society of Pharmacognosy, Okayama, 1985*, Okayama University, Okayama, 1985, Abstract papers, p. 9.
16 T. Okuda, T. Yoshida, T. Hatano, K. Yazaki and M. Ashida, *Phytochemistry*, 21 (1982) 2871.
17 G. Nonaka, S. Morimoto and I. Nishioka, *Chem. Pharm. Bull.*, 34 (1986) 941.
18 T. Okuda, T. Yoshida, T. Hatano, Y. Ikeda, T. Shingu and T. Inoue, *Chem. Pharm. Bull*, in press.