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Review

Counter-current chromatography

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ABSTRACT

The simplicity of counter-current chromatography (CCC) is sometimes overshadowed by a complex description of its mechanical and chromatographic attributes. The universal features of chromatographic theory relevant to CCC are summarized here, using a partition coefficient elution scale to present the chromatogram in a general, readily visualized, format. The principal types of CCC apparatus are summarized, along with selected applications and an indication of the type of apparatus best suited for some specific applications.

CONTENTS

1. INTRODUCTION

Counter-current chromatography (CCC) refers to the process of liquid–liquid chromatography (LLC) carried out without the aid of a solid supporting matrix to retain the stationary liquid in the chromatographic column. Although preceded by early forms of discontinuous counter-current distribution (CCD), CCC can be traced

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to at least 1934, when Cornish *et al.* [1] described a locular CCC column and applied it to the purification of oil-soluble vitamins (see ref. 2).

A renaissance in the development of efficient, laboratory-scale CCC apparatus has followed the description by Ito *et al.* [3] in 1966 of a coil planet centrifuge counter-current chromatograph containing a column consisting of a helical coil of plastic tubing. The principal techniques in current use are: (1) droplet counter-current chromatography (DCCC) [4], (2) centrifugal droplet counter-current chromatography (CDCCC) [5], also referred to as centrifugal partition chromatography (CPC), and (3) CCC with the multilayer coil planet centrifuge (MLCPC) [6]. Reviews of CCC instrumentation and applications have recently been published [1,7–12], including some in this thematic issue of the *Journal of Chromatography*.

The term counter-current chromatography usually refers to the process in which one liquid phase is maintained by gravitational or centrifugal forces as a stationary bed, distributed longitudinally in a column, while a second immiscible liquid phase flows through the stationary bed. However, true counter-current chromatography, in which both phases do flow in opposite directions relative to the columnn, has been carried out in the MLCPC apparatus [13]. The following theoretical discussion, which summarizes a more detailed presentation in ref. 2, refers to the simple CCC process, with one phase stationary.

2. CCC THEORY

Since solute adsorption is precluded by the absence of a solid support, retention in CCC can be accurately predicted from the solute partition coefficient, K, and the relative volumes of mobile and stationary phase, V_m and V_s. The process of CCC is illustrated schematicallyin Fig. 1. A mobile upper phase is illustrated, but, in general, either phase can be employed as mobile phase. The partition coefficient, as is customary in chromatography, is defined as

$$K = C_s/C_m \tag{1}$$

where C_s and C_m represent solute concentrations in stationary and mobile phase respectively.

2.1. The centrality of K = 1

The focal point of the countercurrent chromatogram (and, indeed, of any LLC chromatogram) is the emergence of the solute with a partition coefficient of unity.

Neglecting correction for any extra-column dead volume, solutes with $K = 1$ will always emerge when one column volume, V_c, of mobile phase has passed through the column. If the retention volume of a solute with $K = 1$ is designated as V_1, then $V_1 = V_c$. The elution of $K = 1$ at V_c, which is independent of the relative phase volumes and the flow-rate, is the hub of the counter-current chromatogram.

2.2. Retention times

Non-retained solutes, for which $K = 0$, will have eluted one stationary phase volume, V_s, earlier (Fig. 1B).

Solutes with integral partition coefficients greater than zero are eluted at

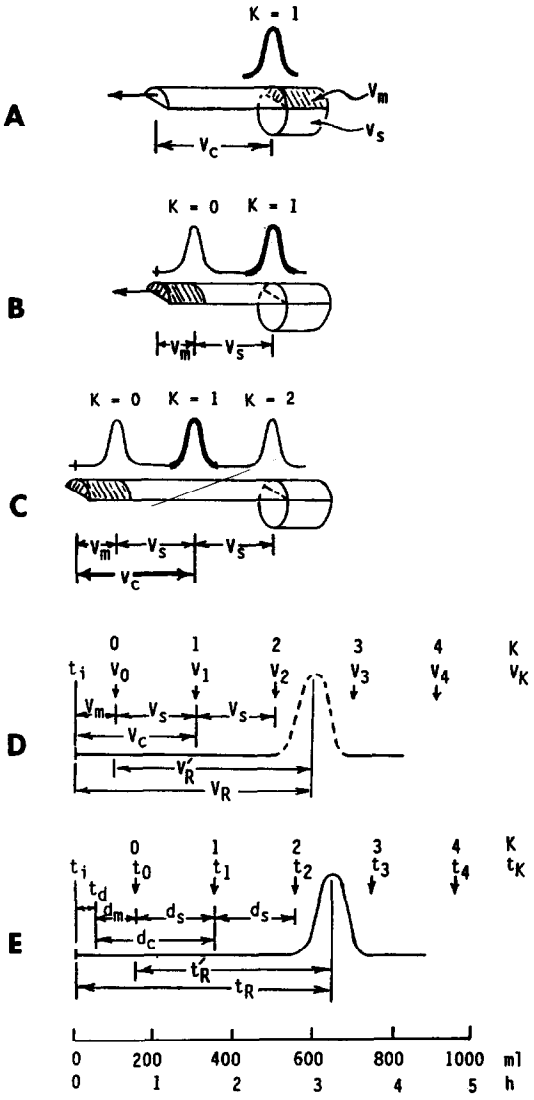


Fig. 1. Generalized counter-current chromatograms. (A, B, C) Elution of solutes with $K = 1, 2$. (D) Indexing the chromatogram by volume, dead volume negligible. (E) Parameters indicated as time and corresponding distances, d, dead volume included. The elution scale corresponds to V_c 300 ml, V_s 200 ml, V_d 50 ml, and flow-rate 200 ml/h.

multiples of the stationary phase volume (Fig. 1C). The general expression for the adjusted retention volume, V_R, of a solute can then be written as

$$V'_R = V_R - V_m - V_d = KV_s \tag{2}$$

where V_R is the retention volume and V_d is a correction for extra-column dead volume, which is usually negligible in preparative applications of CCC.

2.3. Indexing the chromatogram

It is useful to employ the elution volumes of solutes with unit partition coefficients to index the countercurrent chromatogram, as illustrated in Fig. 1D. The symbols V_0, V_1, V_2, ...V_K are convenient. No standards are required. One needs to know only V_c, V_s and, if not negligible, V_d, to fix these points. When a loop is used for sample injection, adding half the injection volume, V_i, to the feed-line volume, V_f, improves the estimate of V_d for large samples, $V_d = V_f + V_i/2$. (If the Ito injection procedure [2] is employed, whereby one injects the sample directly into the column prior to starting mobile phase flow, half the injection volume is subtracted instead as $V_d = V_f - V_i/2$. This will result in a negative V_d for large sample volumes). Partition coefficients of solutes are readily apparent by visual inspection of the indexed chromatogram. For instance, the broken-line peak in Fig. 1D obviously represents a solute with a K of about 2.5.

The chromatogram indexed with a temporal scale is illustrated in Fig. 2E. The corresponding retention times, $t_1, t_2, \ldots t_K$, are obtained by dividing the volumes by the mobile phase flow-rate, f, $t_0 = V_0/f$. The effect of extra-column dead volume in shifting the chromatogram is illustrated in Fig. 1E. The dead time is $t_d = V_d/f$ and t_R and t'_R represent the retention time and adjusted retention time, respectively. The symbols d_m, d_s and d_c represent distances on the chromatogram corresponding to passage of V_m, V_s and V_c volumes of mobile phase, respectively.

Use of the symbols V_0 or t_0 and V_1 or t_1 for indexing is preferable to V_m or t_m and V_c or t_c, respectively, since the latter terms are ambiguous when the system contains dead volume. It is convenient to simply designate the time intervals corresponding to V_m and V_s, on the temporal chromatogram to avoid use of the symbol t_s which is widely used to indicate time spent by solute in the stationary phase.

2.4. Estimating V_1, V_0 and V_s

The position of V_1 is calculated from the known volumes in the system

$$V_1 = V_d + V_c = V_f \pm V_i/2 + V_c \tag{3}$$

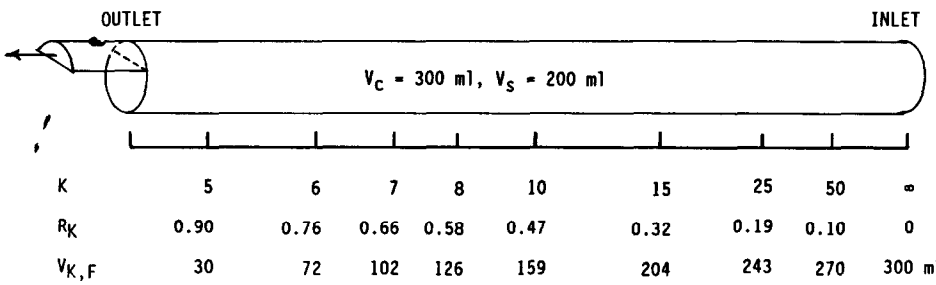
The term $V_i/2$ is + for loop injection but − when the Ito or on-column injection technique is used. In starting up the chromatograph, it is common practice to first fill the column completely with stationary phase. Collection of the volume of stationary phase "carried over", V_{co}, when the mobile phase first passes through the column, provides a measure of the mobile phase volume plus any dead volume in the system, $V_{co} = V_m + V_d \approx V_0$.

Alternatively, one can add a non-retained marker to the sample to obtain a direct indication of V_0.

V_s is obtained by subtracting V_0 from V_1. The positions of V_2, V_3, etc. are then estimated by marking off the distance corresponding to V_s on the chromatogram.

2.5. Solutes retained in the column

A major advantage of CCC is the ability to easily recover solutes retained in the stationary phase by extruding the column contents. The longitudinal position of



K	5	6	7	8	10	15	25	50	∞
R_K	0.90	0.76	0.66	0.58	0.47	0.32	0.19	0.10	0
$V_{K,F}$	30	72	102	126	159	204	243	270	300 ml

Fig. 2. Distribution of retained solutes along the column. Illustrated for the 300-ml column, with V_s of 200 ml, in Fig. 1D, after passage of 1000 ml of mobile phase.

solutes of partition coefficient K, within the column, is indicated by the parameter R_K, which is similar to the R_F value in planar chromatography. R_K is calculated as

$$R_K = \frac{d_K}{L} = \frac{C}{1 + S_F(K - 1)} \tag{4}$$

where S_F indicates the fraction of column volume occupied by stationary phase,

$$S_F = V_s/V_c \tag{5}$$

and C is the number of column volumes of mobile phase passed through the column. R_K is the distance, d_K, moved by the solute with partition coefficient K, divided by the column length, L. An example corresponding to the chromatogram in Fig. 1D, following development with 1000 ml of mobile phase, is given in Fig. 2. Solutes recovered by extruding the column in the forward direction (the direction of development) may be characterized by their forward extrusion volumes, $V_{K,F}$,

$$V_{K,F} = (1 - R_K)V_c \tag{6}$$

some of which are indicated in Fig. 3 for a 300-ml column with V_s of 200 ml.

2.6. Resolution

The contributions of the separation factor, α, column efficiency, N, partition coefficient, K, and stationary phase fraction, S_F, to resolution, R_s, are summarized in the equation

$$R_s = \tfrac{1}{4}(\alpha - 1)\sqrt{N}\ \frac{K_1}{K_1\left(\frac{\alpha + 1}{2}\right) + \left(\frac{1 - S_F}{S_F}\right)} \tag{7}$$

where K_1 is the partition coefficient of the first solute of the pair to be eluted. The separation factor is defined as $\alpha = K_2/K_1$ and will, therefore, always be greater than unity. The first part of the equation is identical to the Knox equation, which is commonly used to describe resolution in high-performance liquid chromatography. However, the bracketed term indicates that the fraction of stationary phase, S_F, in the

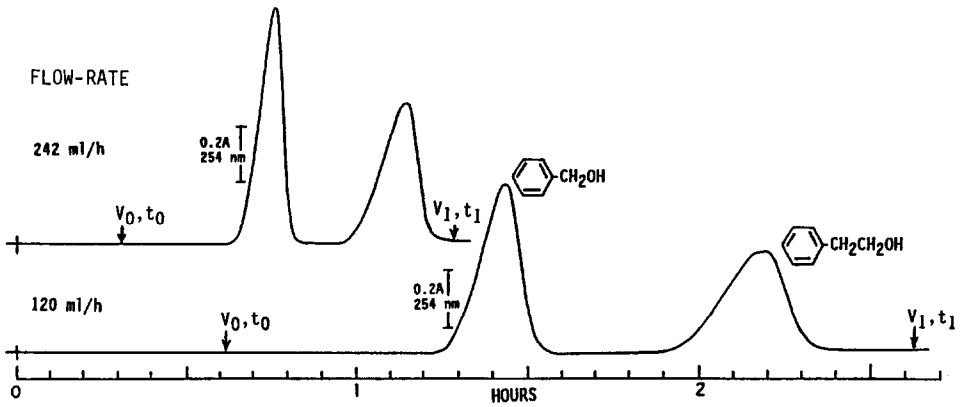


Fig. 3. CCC separation of 20 mg each of benzyl alcohol and phenylethanol, showing the effect of mobile phase flow-rate. Solvent system is heptane–(25% 2-propanol in water) (1:1, v/v) with a mobile aqueous phase. Instrument is the P.C. Inc. MLCPC with a 1.68 mm PTFE column, V_c 315 ml. Adapted from Fig. 9-4 of ref. 2.

column makes a major contribution to resolution. The ratio $(1 - S_F)/S_F$ is equivalent to the phase volume ratio V_m/V_s. The effect of changing V_s can be visually ascertained by examining Fig. 1C. If V_s is imagined to decrease, as may occur when the mobile phase flow-rate is increased, V_m will increase, bringing $K = 0$ closer to the invariant position of $K = 1$. At the same time, solutes with higher K values also approach $K = 1$ until, in the extreme, when $V_s = 0$, the column contains only a single phase and all solutes emerge at V_c. On the other hand, as V_s is increased, the corresponding distance on the chromatogram, representing resolution, increases, approaching a maximum as V_s approaches the column volume V_c, corresponding to S_F approaching unity. With modern apparatus, S_F is in the range of 0.5 to 0.9.

CCC typically provides an efficiency, N of 350 to 1000 theoretical plates. As in other forms of chromatography, selectivity, $\alpha = K_2/K_1$, which is determined by the solvent system, contributes dramatically to resolution. An advantage of CCC is its ability to employ a wide range of solvent systems.

The separation of benzyl alcohol and phenylethanol, shown in Fig. 3, illustrates the resolving power of CCC for a 40-mg sample. A sample of 800 mg is still baseline resolved at the slower flow-rate. Peak symmetry is much better than that obtained in adsorption chromatography.

2.7. Choosing a solvent system

The predictability of CCC performance makes it advantageous to search for solvent systems by measuring K values of sample constituents using analytical techniques other than CCC itself. A micro shake-flask approach coupled with thin-layer chromatography densitometry, high-performance liquid chromatography, gas–liquid chromatography or simple ultraviolet absorptiometry is generally convenient. Aiming for a system providing a K of 1 for the solute of interest is usually desirable. A K of 1 normally provides adequate resolution from more and less polar components but still permits chromatography in a reasonable time. With a typical column volume of 300 to 400 ml and a flow-rate in the range of 100 to 200 ml/h, a solute

with $K = 1$ requires 1.5 to 4 h for elution. As seen from Fig. 1E, it is impractical to elute components with K greater than about 3 within a typical workday. These components can still be recovered either by extrusion of the column contents or by inverting the mode of chromatography by pumping the formerly stationary phase in the anti-development direction. Comparable flow-rates can be employed in centrifugal apparatus of the MLCPC and CDCCC type, but permissible flow-rates in gravitational DCCC apparatus are much slower, requiring separation times up to about 72 h, though still providing good resolution.

The gravitational DCCC system is limited to solvents which form droplets. Various mixtures of chloroform–methanol–water have been most widely used [14,15]. The centrifugal apparatus do not require droplet formation and accommodate a broader variety of solvent systems, ranging in polarity from hexane–water to ethyl acetate–water, 1-butanol–water and 2-butanol–water. Solute partition coefficients can be systematically shifted by varying the content of a third or fourth component. The system hexane–ethyl acetate–methanol–water, which forms two phases over a wide range of compositions, is particularly versatile. Partitioning of solutes into the organic layer is usually favored by increasing the ethyl acetate content, while transfer to the aqueous phase is promoted by adding methanol. Since hexane and water are readily saturated by many organic compounds, sample capacity is usually greater in systems high in ethyl acetate and methanol.

2.8. The effect of pH

Variation in pH can be exploited to shift the partition coefficients of compounds which ionize. In the region where they are ionized (above the pK_a for acids, below the pK_a for bases), the partition coefficient can be expected to change 10-fold for a unit change in pH. Therefore pH must be carefully controlled and sufficient buffering capacity must be provided to accommodate the solute concentration encountered when large amounts of sample are chromatographed.

2.9. Polar and non-polar compounds

CCC offers obvious advantages for polar samples, which do not dissolve in common high-performance liquid chromatography solvent systems, and for labile compounds, which might be altered or adsorbed on contact with silica or other solid supports. The technique is therefore widely used by investigators in the field of natural products, including antibiotics.

CCC is equally applicable to lipophilic compounds. Of many organic solvents immiscible with hexane, the hexane–methanol and hexane–acetonitrile systems are most widely used. The latter system was recently applied to the CCC separation of a 300-g mixture of the ethyl esters of steric, oleic, linolenic and linoleic acids [16]. Formamide and ethylene glycol exhibit good dissolution properties for organic compounds. They function well as the stationary phase in CCC. Formamide forms two-phase systems with solvents as polar as diethyl ether, and ethylene glycol is relatively immiscible with ethyl acetate as well. The system ethylene glycol–chloroform was recently used to isolate 2,4-dinitrophenylvaleric acid from a crude reaction mixture [17]. Traces of ethylene glycol in the chloroform eluent were easily removed by washing the fractions with water prior to evaporation.

Crude sample mixtures, which might readily foul a high-performance liquid

chromatography column, are acceptable in CCC. Good solubility in both phases is required for high sample capacity, since the limit will be reached as saturation is approached in the phase providing lower solubility. A capacity of about 1 g is commonly obtained with a 400-ml column by dissolving the sample in up to 30 ml of either phase or in a mixture of both phases. Better resolution is obtained with a smaller sample volume.

2.10. Normal-phase or reversed-phase CCC

Most solvent systems permit the choice of either phase as the mobile phase. Both aqueous and non-aqueous systems employing the more polar phase as stationary phase can be considered to represent normal-phase CCC. The converse, with the less polar phase stationary, corresponds to the reversed-phase mode.

To simplify application of the chromatographic equations, in which K is defined as C_s/C_m (eqn. 1), it is convenient to define two partition coefficients:

$$K_N = C_{\text{non-polar}}/C_{\text{polar}} \tag{8}$$

and

$$K_P = C_{\text{polar}}/C_{\text{non-polar}} = 1/K_N \tag{9}$$

These symbols apply equally well to aqueous and non-aqueous systems. The subscript N for non-polar also infers the non-aqueous phase in aqueous systems, where water is always the more polar phase. The appropriate partition coefficient for application of the retention equation (eqn. 2) or the resolution equation (eqn. 7) will then be K_N for the reversed-phase mode (nonpolar stationary phase) and K_P for the normal-phase mode (polar stationary phase).

In cases where the chromatographer has a choice of either the normal- or reversed-phase chromatographic mode, eqn. 7 predicts that resolution of a particular pair of solutes will be greater for the mode in which the first solute to be eluted has the higher partition coefficient. The order of elution is, of course, reversed in the two modes. Note that the value of α will be the same in either mode and the judgement just made assumes S_F to be the same in each mode.

For instance, if the partition coefficients for the solutes 1 and 2 are $K_{N1} = 0.5$ and $K_{N2} = 1.0$, then $K_{P1} = 1/0.5 = 2.0$ and $K_{P2} = 1.0$. Since K_{P2} is greater than K_{N1}, eqn. 7 predicts greater resolution in the normal-phase mode, which employs a polar stationary phase.

3. OTHER CCC APPARATUS

This overview summarizes the principles, apparatus and a few applications for the counter-current chromatographic purification of soluble solutes. This use is the most extensively developed at the present time. From its inception however, Ito has demonstrated the applicability of CCC to the separation of a variety of samples. These include particulate (latex particles, cells and subcelluar organelles) separations by sedimentation–elutriation CCC using a single-phase eluent or by partitioning between

two-phase aqueous polymer systems. Proteins can be separated using the latter process as well as by foam CCC.

Ito's extensive research on the optimization of CCC instrumentation has resulted in several devices individually suited for a particular type of sample. For example, a non-synchronous horizontal flow-through coil planet centrifuge is ideal for separation of particulates by sedimentation–elutriation CCC [18,19]. Two-phase aqueous systems have been employed in the non-synchronous CCC as well as in several other units, including the helix CCC and the toroidal coil planet centrifuge [20–24]. Use of two-phase aqueous systems of the salt/polymer type for the separation of proteins has been demonstrated in the MLCPC and CDCCC apparatus and recently in a compact horizontal flow-through coil planet centrifuge with eccentric multilayer coils [25]. Columns have been described which readily adapt MLCPC apparatus to foam CCC [26–28] and true CCC [13,29].

REFERENCES

1 R. E. Cornish, R. C. Archibald, E. A. Murphy and H. M. Evans, *Ind. Eng. Chem.*, 26 (1934) 397.
2 W. D. Conway, *Countercurrent Chromatography Apparatus, Theory and Applications*, VCH, New York, 1990.
3 Y. Ito, M. A. Weinstein, I. Aoki, R. Harada, E. Kimura and K. Nunogaki, *Nature (London)*, 212 (1966) 985.
4 T. Tanimura, J. J. Pisano, Y. Ito and R. L. Bowman, *Science (Washington, D.C.)*, 169 (1970) 54.
5 W. Murayama, T. Kobayashi, Y. Kosuge, H. Yaro, Y. Nunogaki and K. Nunogaki, *J. Chromatogr.*, 239 (1982) 643.
6 Y. Ito, J. Sandlin and W. G. Bowers, *J. Chromatogr.*, 244 (1982) 247.
7 Y. Ito, *CRC Crit. Rev. Anal. Chem.*, 17 (1986) 65.
8 N. B. Mandava and Y. Ito (Editors), *Countercurrent Chromatography, Theory and Practice (Chromatographic Science Series*, Vol. 44), Marcel Dekker, New York, 1988.
9 K. Hostettman, M. Hostettmann and A. Marston, *Preparative Chromatography Techniques, Applications in Natural Product Isolation*, Springer, Berlin, 1986.
10 I. Sutherland, *Lab. Pract.*, 36 (1987) 37.
11 I. Kubo, F. J. Hanke and G. T. Marshall, *J. Liq. Chromatogr.*, 11 (1988) 173.
12 J. B. McAlpine and J. E. Hochlowski, in G. H. Wagman and R. Cooper (Editors), *Natural Products Isolation (Journal of Chromatography Library*, Vol. 43), Elsevier, Amsterdam, 1989, p. 1.
13 Y.-W. Lee, C. E. Cook and Y. Ito, *J. Liq. Chromatogr.*, 11 (1988) 37.
14 K. Hostettmann, *Planta Med.*, 39 (1980) 1.
15 K. Hostettmann, *Naturwissenschaften*, 70 (1983) 1861.
16 W. Murayama, Y. Kosuge, N. Nakara, Y. Nunogaki, K. Nunogaki, J. Cazes and H. Nunogaki, J. Liq. Chromatogr., 11 (1988) 283.
17 W. D. Conway, J. D. Klingman, D. Greco and K. Huh, *J. Chromatogr.*, 404 (1989) 391.
18 Y. Ito, P. Carmeci, R. Bhatnagar, S. B. Leighton and R. Seldon, *Sep. Sci. Technol.*, 15 (1980) 1589.
19 Y. Ito, *J. Biochem. Biophys. Methods*, 5 (1981) 105.
20 Y. Ito and R. L. Bowman, *Anal. Biochem.*, 85 (1978) 614.
21 Y. Ito, *J. Chromatogr.*, 192 (1980) 75.
22 J. M. Van Alstine, R. S. Snyder, L. J. Karr and J. M. Harris, *J. Liq.Chromatogr.*, 8 (1985) 2293.
23 I. A. Sutherland, D. Heywood-Waddington and T. J. Peters, *J. Liq. Chromatogr.*, 8 (1985) 2315.
24 I. A. Sutherland, D. Heywood-Waddington and Y. Ito, *J. Chromatogr.*, 384 (1987) 197.
25 Y. Ito and H. Oka, *J. Chromatogr.*, 457 (1988) 393.
26 Y. Ito, *J. Liq. Chromatogr.*, 8 (1985) 2131.
27 M. Bhatnagar and Y. Ito, *J. Liq. Chromatogr.*, 11 (1988) 21.
28 H. Oka, K. Harada, M. Suzuki, H. Nakazawa and Y. Ito, *Anal. Chem.*, 61 (1989) 1988.
29 Y. W. Lee, Q. C. Fang, Y. Ito and C. E. Cook, *J. Nat. Prod.*, 52 (1989) 706.