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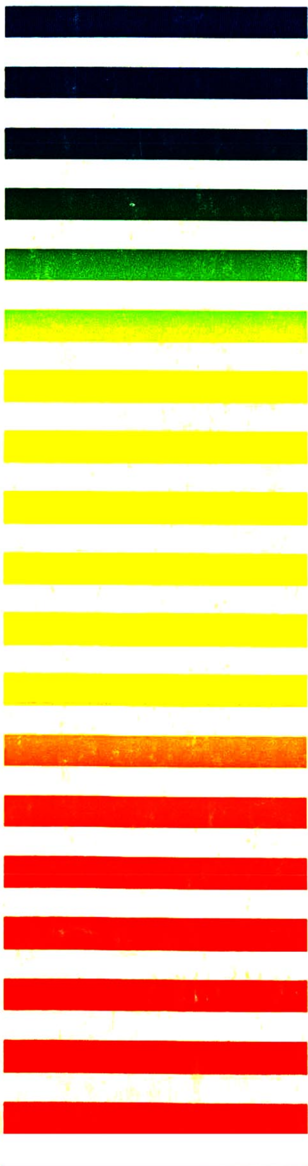

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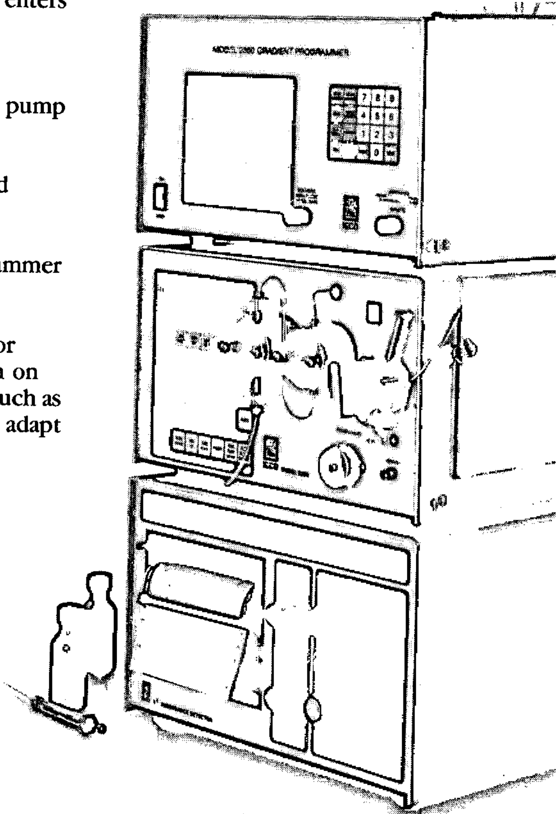

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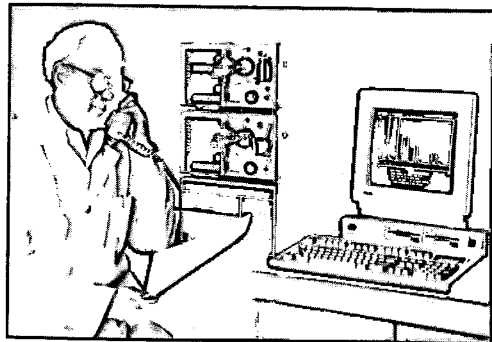

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SECOND INTERNATIONAL SYMPOSIUM ON PREPARATIVE AND UP-SCALE LIQUID CHROMATOGRAPHY

Baden-Baden (F.R.G.), February 1–3, 1988

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FOREWORD

The *Second International Symposium on Preparative and Up-Scale Liquid Chromatography* was held in Baden-Baden, F.R.G., from February 1 to 3, 1988. This symposium, devoted to novel concepts and instrumentation in preparative chromatography, attracted over 420 participants from 25 countries. In view of the growing impact of preparative chromatography in research and production, this symposium provided an excellent forum for chemists, chromatographers, engineers and biochemists to exchange ideas and practical experience.

In the intensive three-day programme the focus was on effective chromatographic procedures for isolating natural and synthetic products, particularly biopolymers. The following aspects were covered: the choice between polymer-type and silica-based packings, the optimum particle size of packings with regard to purity, throughput and cost, the selection of solvents and economical solvent recovery, elution *versus* displacement mode, supercritical fluid *versus* liquid chromatography as alternative means in isolation, purification, single-step and multi-step procedures, recovery of biological activity of isolates, scale-up procedures and process chromatography. As a consequence, this symposium represents an important stage in the development of preparative chromatography as a generally applicable method of purification and isolation. Its vitality was convincingly demonstrated by the high standards of the instrument exhibition, which was integrated into the symposium, with 35 firms from all over the world as exhibitors.

I would like to express my appreciation to all the participants who came to Baden-Baden and made the symposium such a highly successful and enjoyable event. I also wish to express my gratitude to my co-chairmen (D. Bauer, J. F. K. Huber, K.-P. Hupe, J. H. Knox, J. C. Kraak, R. Rosset, M. Verzele and A. Wehrli), who assisted me in the development of the scientific programme. I would also like to acknowledge the efforts of Dr. E. Heftmann, Editor of the *Journal of Chromatography Symposium Volumes*, for handling the manuscripts and of the staff of the *Journal of Chromatography* for their assistance in publishing this special symposium issue. It is a pleasure to acknowledge the excellent support provided for the organization and the management of the symposium by K.-P. Hupe, J. Wendenburg and K. Begitt.

KLAUS K. UNGER

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CHROMSYMP. 1405

ISOLATION OF BIOLOGICALLY ACTIVE PLANT CONSTITUENTS BY LIQUID CHROMATOGRAPHY

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SUMMARY

Strategies are outlined for the separation of biologically active products of plant origin. The techniques involved include low-pressure liquid chromatography, semi-preparative high-performance liquid chromatography, flash chromatography and droplet counter-current chromatography. Their application to the isolation of compounds from *Sesamum angolense* (Pedaliaceae), *Psorospermum febrifugum* (Guttiferae) and *Cordia goetzei* (Boraginaceae) is described.

INTRODUCTION

The search for new compounds from plant sources is an expanding field of research, which often has as its aim the development of pharmacologically active substances. Whereas these phytochemicals may themselves be of interest, they also provide starting materials for the further investigation (by synthesis or partial synthesis) of other molecules with certain desired activities. The isolation of the pure biologically active constituents responsible for the properties of medicinal plants is essential for structure elucidation, structural modifications and for studying the mode of action of the drug, its side-effects, toxicology, etc. For these investigations, quantities in excess of 10 mg of the relevant compound are generally required. The pathway from the plant to the pure material can be long and tedious. After extraction, the active material must be fractionated, each separation step being guided by a convenient assay. Most important of all during this process is the judicious choice of chromatographic techniques for maximum yield of pure substances with a minimum of effort. Some of the preparative separation techniques available[1] are: paper chromatography, preparative thin-layer chromatography (TLC) (and centrifugal TLC), open-column chromatography, vacuum liquid chromatography, pressure column chromatography [flash, low-pressure liquid chromatography (LPLC), medium-pressure LC (MPLC), high-pressure LC (HPLC)], liquid–liquid chromatography [Craig distribution, droplet counter-current chromatography (DCCC), rotation locular counter-current chromatography (RLCC), centrifugal partition chromatography (CPC)].

Although in some cases a single chromatographic step may suffice to isolate the

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required amount of compound, a combination of methods is most often required. A number of strategies are possible for the separation of lipophilic or hydrophilic substances. Some of these will be illustrated here, with special reference to our on-going programme of research on the biologically active compounds from African medicinal plants. For example, a phytochemical investigation of the plant *Sesamum angolense* Welw. (Pedaliaceae) from tropical Africa is presently being undertaken because of the presence of haemostatic and fungicidal[2] compounds in the root bark. Another plant of current interest is *Psorospermum febrifugum* Spach (Guttiferae), extracts of which are specifically toxic to human colon carcinoma cells[3]. Previous phytochemical investigations led to the isolation of the xanthone psorospermin, an antileukaemic principle of this African plant[4]. However, further examination of the light petroleum (b.p. 60–95°C) extract of the root bark has resulted in the isolation of a series of lipophilic anthranoid derivatives which are responsible for the major part of the cytotoxic activity[3]. Finally, *Cordia goetzei* Gürke (Boraginaceae) is a medicinal plant used in Tanzania for the treatment of leprosy, malaria and abscesses. The stem bark of this tree contains a mixture of fungicidal highly oxygenated polyphenols[5].

EXPERIMENTAL

Iridoid glycosides and a phenylpropanoid glycoside from Sesamum angolense (Pedaliaceae)

Extraction of *Sesamum angolense* root bark from Malawi was carried out first with dichloromethane and then with methanol. DCCC of the methanol extract on a Büchi 670 instrument (Flawil, Switzerland) with chloroform–methanol–2-propanol–water (5:6:1:4) in the ascending mode yielded 15 fractions (I–XV). Preparative LPLC of fraction IV on a Lobar size B column (LiChroprep RP-8, 40–63 μm) (Merck, Darmstadt, F.R.G.), equipped with a Duramat-80 pump (Chemie und Filter, Regensdorf, Switzerland), Uvicord SII detector (254 nm) (LKB, Bromma, Sweden) and LKB 2210 chart recorder, yielded compounds 1 and 2 (Fig. 1) on elution with 10% aqueous methanol. Subsequent elution with 32% aqueous methanol gave compound 3. The same method gave compound 4 from fraction VII and compound 5 from fraction XI, with 15% aqueous methanol as the eluent. Full details of the structure elucidation for compounds 1–5 have been reported elsewhere[6].

Analytical HPLC was performed with 5-μl samples on a 7-μm LiChrosorb RP-8 column (250 mm × 4.6 mm I.D.) (Knauer, Bad Homburg, F.R.G.) with 10% aqueous methanol (Fig. 2A) or 32% aqueous methanol (Fig. 2B) at a flow-rate of 1.5 ml/min (Model 8700; Spectra-Physics, San Jose, CA, U.S.A.). Detection was at 254 nm (LKB Uvicord SII).

Anthracene derivatives of Psorospermum febrifugum (Guttiferae)

Root bark of *Psorospermum febrifugum*, collected in Malawi, was extracted with light petroleum (b.p. 60–95°C). The resulting orange resin was cytotoxic to a Co-115 human colon carcinoma cell line[3] and this assay was employed for subsequent fractionation of the extract. Full details of the isolation and structure determination of compounds 6–11 (Fig. 4) from the light petroleum extract have been given elsewhere[3]. Flash chromatography was performed on silica gel Si 60 (63–200 μm) (Merck) and gel filtration on Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Preparative LPLC

separations of compounds 7, 10 and 11 was carried out on Lobar size B columns (LiChroprep RP-8, 40–63 μm) with 93% aqueous methanol at a flow-rate of 2 ml/min.

Analytical HPLC was carried out with 10 μl of a 5 mg/ml solution of an extract on a 7-μm LiChrosorb RP-18 column (250 mm × 4.6 mm I.D.). The eluent (Fig. 5) was delivered at 1.5 ml/min by a Spectra-Physics 8700 pump. Chromatograms and spectra were recorded with an HP1040A photodiode-array detector (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Polyphenols from Cordia goetzei (Boraginaceae)

Stem bark of *Cordia goetzei* was extracted sequentially with light petroleum (b.p. 60–95°C) chloroform and methanol. Screening for biological activity with a TLC bioassay[7] showed the presence in the methanol extract of compounds active against the fungus *Cladosporium cucumerinum*. Subsequent fractionation of the methanol extract by DCCC on a Büchi 670 apparatus with chloroform–methanol–water (43:37:20) in the descending mode and crystallization of fractions 26–35 from ethyl acetate–hexane gave compound 14. Polyphenol 15 was obtained after subjecting DCCC fractions 39–43 to LPLC on a Lobar size B column of LiChroprep RP-8 (40–63 μm) with 60% aqueous methanol. Full details of the isolation of compounds 14 and 15, together with their structure determination, have been given elsewhere[5]. Compounds 12 and 13 were separated by semi-preparative HPLC of DCCC fractions 161–225 on a 10-μm μBondapak C_{18} column (300 mm × 7.8 mm I.D.) (Waters-Millipore, Milford, MA, U.S.A.) with 40% aqueous methanol at a flow-rate of 5 ml/min. The instrumentation comprised a Waters 6000A pump coupled to a Waters automatic gradient controller. Detection at 254 nm was effected with a variable-wavelength UV detector (Pye-Unicam, Cambridge, U.K.). The sample (150 mg) was dissolved in 1 ml of mobile phase and filtered through Millex HV_4 filter units (0.45 μm; Millipore, Milford, MA, U.S.A.). For each batch, 20–50 μl of solution were injected. The structure determination of compounds 12 and 13 has been given elsewhere[5]. Analytical HPLC was carried out with 5 μl of a 5 mg/ml solution on 7-μm LiChrosorb RP-18 (250 mm × 4.6 mm I.D.) or 10-μm μBondapak C_{18} (300 mm × 3.9 mm I.D.) columns. The eluent (the composition is shown in Figs. 7 and 8) was delivered at 1.5 ml/min by a Spectra-Physics 8700 pump and chromatograms were recorded on an HP1040A photodiode-array detector.

RESULTS

Iridoid and phenylpropanoid glycosides from Sesamum angolense

Following extraction of the root bark of *Sesamum angolense* with dichloromethane, the methanol extract was found to contain a number of iridoid glycosides. In order to obtain the pure glycosides, the methanol extract was first subjected to a DCCC fractionation step. Analytical HPLC of fraction IV on RP-8 with 10% aqueous methanol (Fig. 2A) allowed the separation of the iridoid glycosides phlomiol (1) and pulchelloside-I (2) within 13 min. Elution with 32% aqueous methanol (Fig. 2B) gave an additional peak after *ca.* 12 min, correponding to 3 (verbascoside). However, in this case, compounds 1 and 2 were not separated. The preparative separation of compounds 1–3 was performed by LPLC (Fig. 3). The analytical conditions can be directly applied to the large-scale method. Thus, after injection of

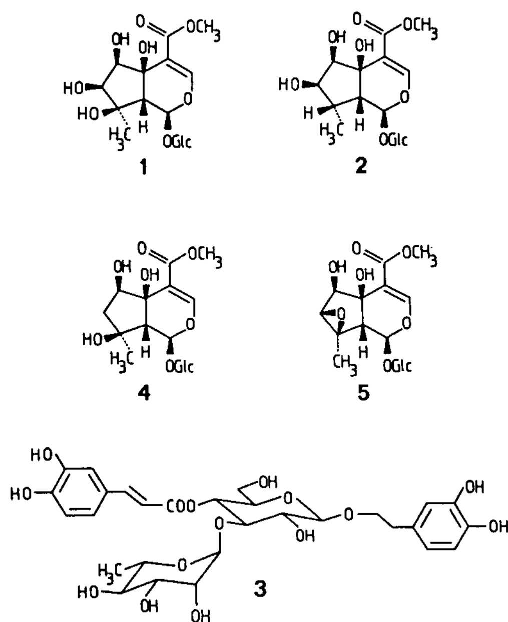

Fig. 1. Glycosidic components of the root bark of *Sesamum angolense* (Pedaliaceae). Glc = Glucose.

130 mg of sample, elution with 10% aqueous methanol enabled the isolation of compounds 1 (71 mg) and 2 (9 mg). Increasing the methanol content of the eluent to 32% gave, in addition, 10 mg of verbascoside. The separation was complete in 20 h.

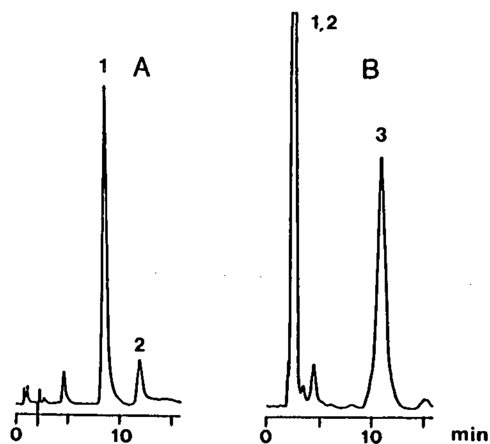

Fig. 2. HPLC analysis of DCCC fraction IV from the methanol extract of *Sesamum angolense* root bark. Column: LiChrosorb RP-8. Detection: 254 nm. Flow-rate: 1.5 ml/min. Sample: 5-μl (25 μg). Eluents: A, 10% aqueous methanol; B, 32% aqueous methanol.

Fig. 3. LPLC separation of DCCC fraction IV from the methanol extract of *Sesamum angolense* root bark. Column: Lobar RP-8 (310 mm × 25 mm). Eluent: aqueous methanol (MeOH, 10 to 32%) step gradient; flow-rate, 1.5 ml/min. Detection: 254 nm. Sample: 130 mg (3 ml).

Compound 4 (6β-hydroxyipolamiide) and the new iridoid glycoside sesamoside (5) were similarly obtained by the combination of DCCC with LPLC.

Cytotoxic anthracene derivatives from Psorospermum febrifugum

An analytical separation of the major anthranoid pigments occurring in the root bark of *Psorospermum febrifugum* is shown in Fig. 5. Fractionation of the light petroleum extract gave a total of six yellow compounds 6–11 (Fig. 4). Compounds 6,

Fig. 4. Anthranoid pigments obtained from *Psorospermum febrifugum* (Guttiferae).

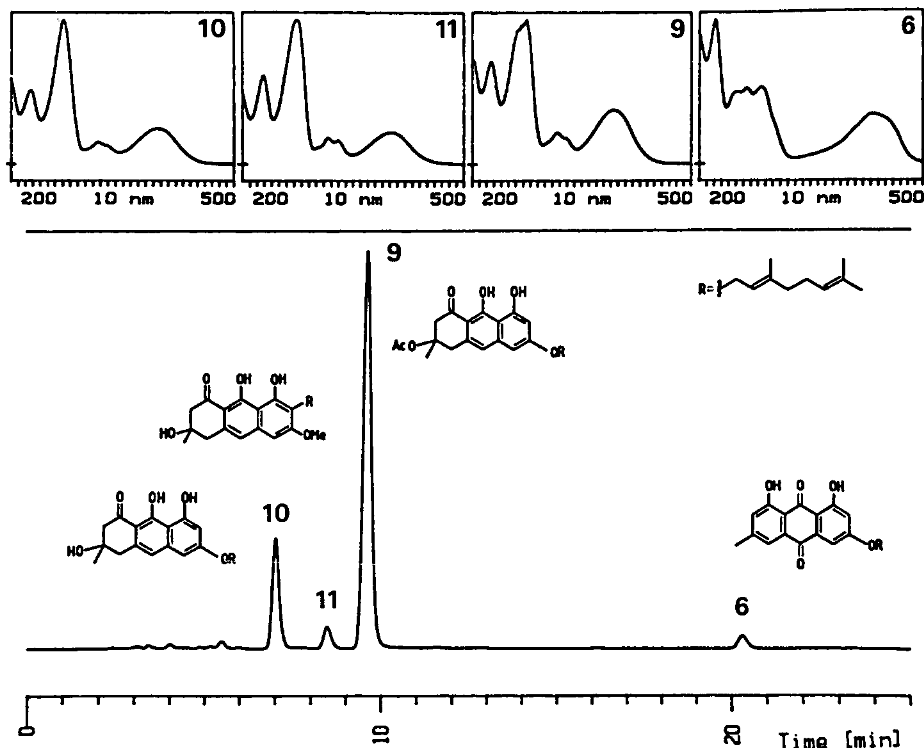

Fig. 5. HPLC–UV analysis of a light petroleum extract of *Psorospermum febrifugum* root bark. Column: LiChrosorb RP-18. Eluent, 90 to 100% methanol (+ 0.5 ml/l phosphoric acid) over 15 min, then 100% methanol for 10 min; flow-rate, 1.5 ml/min. Detection: 254 nm. Sample: 10 μl (50 μg). Me = Methyl; Ac = acetyl.

8 and 9 were crystallized directly from the relevant flash chromatography fractions[3]. Anthrone (8) although previously described as occurring in the plant[8], was obtained only as an artefact of isolation and was not observed in the HPLC analysis of the extract. Traditional chromatographic methods, such as open-column chromatography, led to decomposition and irreversible adsorption of the sensitive pigments. It was necessary, therefore, to use rapid (flash and low-pressure liquid chromatography) or weakly interactive (gel filtration) methods for the successful isolation of the cytotoxic compounds. The tetrahydroanthracenes 9 and 10 were the major cytotoxic constituents of *P. febrifugum* root bark. Tetrahydroanthracene (11)[9] was also active but anthrone (8) only exhibited marginal cytotoxicity. Anthraquinone (7) was isolated in only small amounts from the extract and, like anthraquinone 6, was biologically inactive.

Antifungal polyphenols from Cordia goetzei

The antifungal methanol extract of *Cordia goetzei* stem bark was found to be a very complex mixture when analysed by HPLC-UV (Fig. 7). Preliminary fractionation by DCCC, together with a bio-autographic TLC assay for fungicidal activity[7], enabled the attribution of activity to a certain number of defined components of the

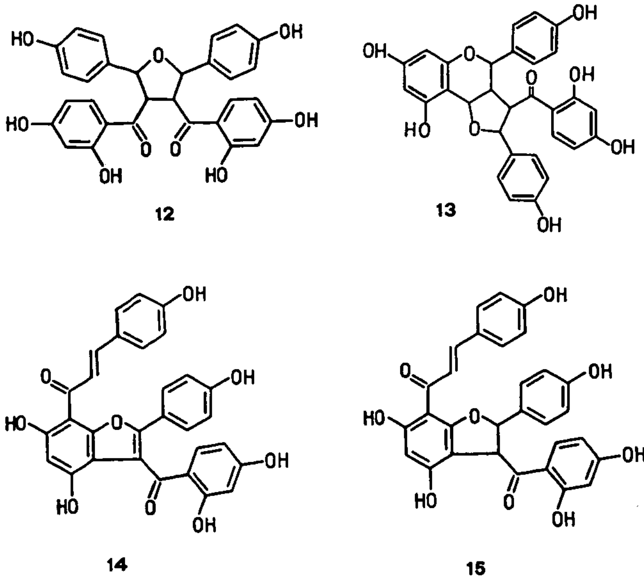

Fig. 6. Antifungal polyphenols from *Cordia goetzei*.

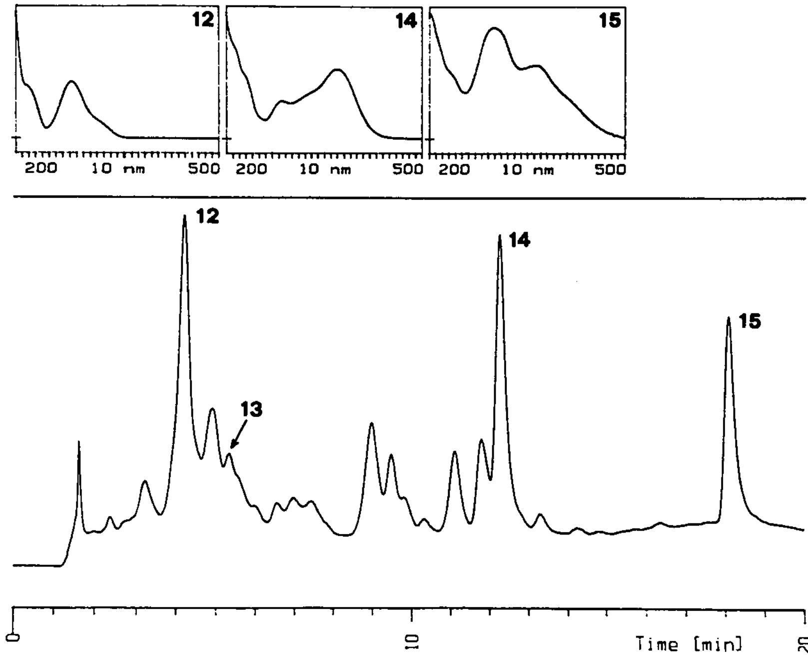

Fig. 7. HPLC–UV analysis of a methanol extract of *Cordia goetzei* stem bark. Column: LiChrosorb RP-18. Eluent: 55 to 75% methanol over 15 min, then 75% methanol for 5 min; flow-rate, 1.5 ml/min. Detection: 254 nm. Sample: 5 μl (50 μg).

mixture. Thus, DCCC fractions 161–225 were shown to contain two novel major antifungal compounds, 12 and 13. An analytical HPLC separation of compounds 12 and 13 on an octadecylsilyl column is shown in Fig. 8, together with the corresponding UV spectra, recorded on-line with a photodiode-array detector[10]. The analytical HPLC separation was used as the basis for determining semi-preparative HPLC separation conditions. A typical semi-preparative HPLC chromatogram on a μBondapak C_{18} column with 40% aqueous methanol as eluent is shown in Fig. 9. Baseline separation of compound 12 occurred, but 13 had to be collected by heart-cutting in order to obtain the pure product. By repetitive injections, a total of 83 mg of compound 12 and 29 mg of compound 13 were obtained. These two compounds were indistinguishable by TLC on silica gel in several solvent systems.

In addition, two known benzofuran derivatives, 14 and 15, were isolated from the stem bark[5]. The antifungal compound 14 was purified by direct crystallization after DCCC, whereas the antifungal dihydrobenzofuran 15 required an additional LPLC step on an RP-8 column for final purification.

CONCLUSIONS

Some of the strategies employed for the separation of biologically active substances from medicinal plants have been described. A liquid–liquid partition technique (DCCC), followed by LPLC on reversed-phase columns, enabled the

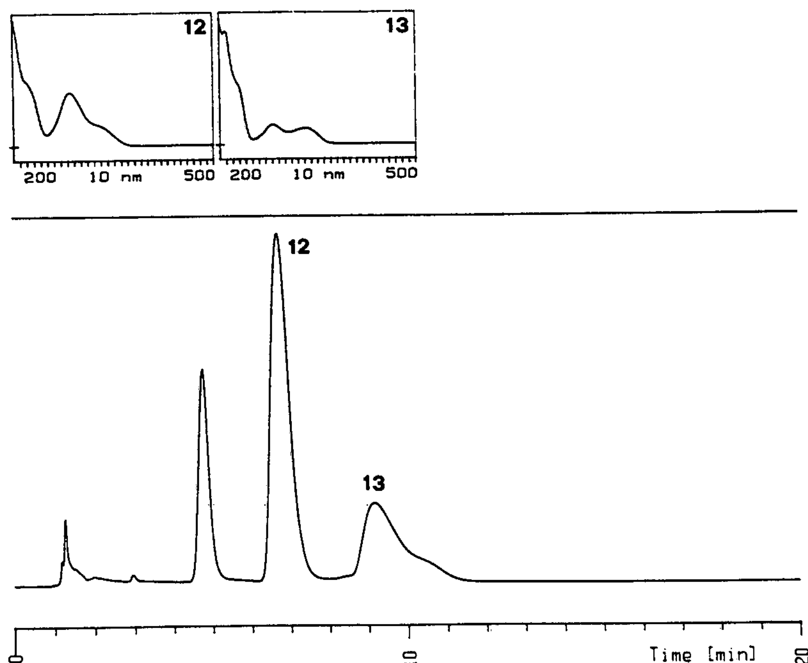

Fig. 8. HPLC–UV analysis of DCCC fractions 161–225 from the methanol extract of *Cordia goetzei* stem bark. Column: μBondapak C_{18} (300 mm × 3.9 mm). Eluent: 50% methanol; flow-rate, 1.5 ml/min. Detection: 254 nm. Sample: 5 μl (25 μg).

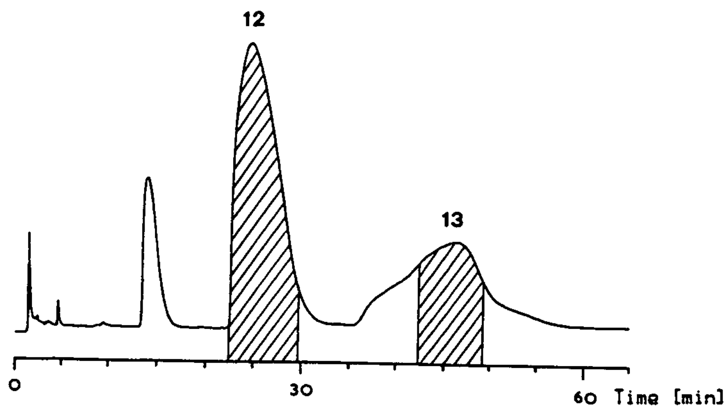

Fig. 9. Semi-preparative HPLC separation of compounds 12 and 13 from *Cordia goetzei*. Column: μBondapak C_{18} (300 mm × 7.8 mm). Eluent: 40% methanol; flow-rate, 5 ml/min. Detection: 254 nm. Sample: 20 μl of a 150 mg/ml solution.

isolation of iridoid and phenylpropanoid glycosides, and of complex polyphenols. In addition, chalcone-derived polyphenols from *Cordia goetzei* have been separated by semi-preparative HPLC, a chromatographic technique used previously by us in, *e.g.*, the separation of isomeric saponins and closely related chromenes and dichromenes[11]. For more lipophilic compounds, a combination of flash chromatography and LPLC has been used with success for the separation of sensitive cytotoxic constituents of *Psorospermum febrifugum*. Traditional open-column methods led to irreversible adsorption and decomposition of the readily oxidized tetrahydroanthracene derivatives.

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CHROMSYMP. 1420

ANALYSIS OF THE INFORMATION IN A PREPARATIVE CHROMATOGRAM FOR FURTHER OPTIMIZATION OF THE OPERATING CONDITIONS

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SUMMARY

It was demonstrated how the most salient theoretical aspects of linear and non-linear elution liquid chromatography can be used, without the aid of sophisticated optimization software, to analyse the information in a preparative chromatogram. It is important, first, to be able to recognize whether a given preparative chromatogram was obtained under volume- or mass-overload conditions or both. This permits further optimization of the injection conditions (size, volume and concentration of the sample), depending on the preparative objectives, and improvement of the stationary phase particle size and of the mobile phase flow-rate. This approach can also provide insight into the performance characteristics of the injection device, the efficiency of the column packing and the stationary phase capacity. Some examples taken from the recent literature are discussed.

INTRODUCTION

The many systematic studies undertaken in the past few years in all areas of preparative liquid chromatography (PLC) have resulted in greater understanding of the behaviour of preparative columns. It can now be said that the well established theory of linear elution satisfactorily describes separations obtained under volume-overload conditions[1–5]. As it is known that the best performance from a preparative point of view (production rate at a given purity) is often obtained under mass-overload conditions, several theoretical approaches describing the shape of non-linear elution peaks have recently been developed. Most often, the mass-balance differential equation for a single solute has been considered together with a non-linear isotherm. The resulting set of equations has been solved either analytically, in the case of slight isotherm curvature[6–9] (so-called Haarhof–Van der Linde treatment), or numerically, without any restriction[10–21]. The plate model scheme, associated with a Langmuirian isotherm, has also been used to calculate the non-linear elution profile of one solute[13,14] and, lately, that of two unresolved compounds[15,16]. It appears that the Haarhof–Van der Linde treatment is limited to describing slight mass-overload conditions, whereas the other models, the promising preparative applications of which

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are still underway, require sophisticated computer programs. This is why we think that the most straightforward and simplest approach to non-linear PLC at this time is to make use of the semi-empirical model we have developed previously[17] in spite of its inherent limitations. This model was based on a mathematical characterization of strongly non-linear experimental elution peaks[18].

The purpose of this report is to show how some of that linear and non-linear theoretical framework can be used in a very simple practical way to analyse the information in a preparative chromatogram. This analysis will lead to a discussion of operating conditions and, subsequently, to their optimization. This will be simply exemplified by some PLC separations taken from the recent literature, but first the salient theoretical features used in the subsequent discussions will be briefly reviewed.

THEORETICAL

Linearity test

When the injection parameters (quantity, volume and concentration of the sample) are varied, a chromatographic column may present two distinct types of behaviour, which are defined in analogy with the theory of systems[17,19,20]. The linear behaviour corresponds to the usual injection conditions of analytical chromatography and to conditions where the sample volume is the only factor accounting for a peak shape alteration. These conditions are referred to as volume overload in preparative chromatography. The non-linear behaviour corresponds to the other cases in which the peak shape alterations are mainly caused by the curvature of the distribution isotherms (mass overload conditions). While optimizing the operating parameters for preparative purposes, it is of prime importance to characterize the column behaviour for any given experimental injection, because the choice of the optimum conditions is very dependent on whether the column behaves linearly or not. In practice, this characterization can be performed very easily by using a test based on the additivity of the independent contributions to the statistical moments of chromatographic peaks[19]. The procedure consists in checking the following conditions

$V_0/2 \ll V_R$ and $V_0^2/12 \ll \sigma^2$ Linear behaviour (pulse injection)
$V_R' - V_0/2 = V_R$ and $\sigma'^2 - V_0^2/12 = \sigma^2$ Linear behaviour (plug injection)
$V_R' - V_0/2 < V_R$ or $\sigma'^2 - V_0^2/12 > \sigma^2$ Non-linear behaviour

where V_R and σ are the retention volume and standard deviation, respectively, for small-size, small-volume injections, V_R' and σ' are the retention volume and standard deviation, respectively, under the injection conditions being tested and V_0 is the sample volume.

Optimization of injection conditions

For a pair of compounds to be separated on a preparative scale the optimum sample volume can be predicted theoretically. If the resolution of the analytical separation is less than 1.3, neither volume nor mass overload is advocated. The sample volume should not exceed the standard deviation of the narrowest peak of the pair[4]. If the analytical resolution is greater than 1.3, the maximum sample volume, $V_{0,\text{lin}}$, allowing a total recovery in the case of a linear behaviour of the column is given by[1–5]

$$V_{0,\text{lin}} = V_{R_2} - V_{R_1} - 2(\sigma_1 + \sigma_2) \quad (1)$$

in which V_{R_1} and V_{R_2} are the analytical retention volumes of the two compounds and σ_1 and σ_2 the corresponding standard deviations. The maximum sample concentration, $C_{0,\text{lin}}$, consistent with a linear column behaviour, is dependent on the solute retention, molecular size and stationary-phase capacity. Systematic investigations[21] have shown that, in most cases, $C_{0,\text{lin}}$ lies between $5 \cdot 10^{-3}$ and $2 \cdot 10^{-2}$ *M*. This linear optimization procedure leads to operation under volume overload conditions; it is advocated when the column cannot be operated under mass overload, as discussed below.

If the analytical resolution is rather large and the sample solubility high, a non-linear optimization procedure should be followed. The optimum quantity, $Q_{0,2}$, of the more strongly retained of the two compounds of interest can be assessed by using a model based on the experimental characterization of the shape of strongly non-linear elution peaks[21]

$$Q_{0,2} = C_{\text{m},2}\tau_2 \exp\left(\frac{V_{R_1} + 2\sigma_1 - V_\text{m}}{\tau_2}\right) \tag{2}$$

where V_m is the column dead-volume and $C_{\text{m},2}$ and τ_2 the model parameters, which can be evaluated as follows: τ_2 is roughly equal to 0.2 V_{R_2} and $C_{\text{m},2}$ in most cases lies in the range of 0.2–0.8 *M* in the inverse order of solute retention[18]. In addition, it has been observed that this quantity is best injected in a small volume of concentrated solution[21].

DISCUSSION

Mass- and volume overload excluded

We will first exemplify the practical use of the linearity test described above for a separation of two isomeric azo compounds (MW = *ca.* 350) obtained by synthesis[22]. This separation was performed in the reversed-phase mode on Merck Lobar (31 cm × 2.5 cm I.D.) preparative column. The capacity factors of the compounds of interest were determined to be 10.1 and 14.4, and the corresponding analytical resolution was *ca.* 1.2. The effect of sample volume was studied up to 100 ml for a constant sample size (5 mg). It was experimentally determined that the sample volume has no influence on the peak width up to about 70 ml. Table I gives the results obtained for the two extreme values of sample volumes studied, *i.e.*, 10 and 100 ml. Using the methodology mentioned above for linearity testing, it is clear that the chromatographic behaviour of the column under these conditions is linear for these compounds over this range of sample volumes. The chromatogram resulting from a 10-ml injection can be considered as an analytical type impulse response of the column ($V_0/\sigma \approx 0.18$), while the one resulting from a 100-ml injection is a linear chromatographic response to a plug-shaped injection ($V_0/\sigma \approx 1.8$). These results were quite predictable for such a sample size (5 mg, or *ca.* 0.07 mg sample per g stationary phase) and for sample concentrations in the range of from $1.4 \cdot 10^{-4}$ to $1.4 \cdot 10^{-3}$ *M*, *i.e.*, below the usual values for $C_{0,\text{lin}}$. Owing to the resolution of 1.2, the sample volume, for practical preparative purposes, should not be much in excess of the analytical standard deviation of the narrowest peak, *i.e.*, 60–70 ml. This is in agreement with the experimental results reported and means that the injection device used for large

TABLE I

EFFECT OF THE SAMPLE VOLUME, V_0, ON THE SEPARATION CHARACTERISTICS OF TWO ISOMERIC AZO COMPOUNDS

V'_R, σ' = measured retention volumes and standard deviations. Operating conditions: column, 31 cm × 2.5 cm I.D. (Lobar, Merck); stationary phase, LiChroprep RP 8, 50 μm; mobile phase, methanol–water (3:2); flow-rate, 7.4 ml/min; sample size, 5 mg mixture.

	Solute	*Sample volume (ml)*	
		10	*100*
$V'_R - V_0/2$ (ml)	1	804	804
	2	1103	1096
$(\sigma'^2 - V_0^2/12)^{\frac{1}{2}}$ (ml)	1	56	56
	2	68	70

volumes in this study was working quite well. Despite the rather high capacity factors, the sample concentration could probably be raised to around 2–3 · 10^{-3} M, barring solubility limitation, without serious mass-overload effects, and this would allow injections of the order of 50 mg of sample.

Analytical to preparative column scale-up. Mass overload

A second example taken from the literature[23] is the normal-phase separation of two positional isomers, the 1- and 4-hydroxy-1,2,3,4-tetrahydrophenanthrenes (MW = 197). The mobile phase was first optimized for preparative application by considering the criteria of separation selectivity, sample solubility, and volatility, viscosity and consumption of solvents. A dichloromethane–ethyl acetate (95:5) mixture was selected. The resulting analytical chromatogram is shown in Fig. 1A. The analytical column (30 cm × 4.2 mm I.D.) was next used to determine the optimum sample load experimentally, according to the preparative objectives of high purity and yield of both compounds. The result is shown in Fig. 1B. Then, the separation was scaled up to a 10-g sample load on a Waters Prep LC 500 preparative chromatograph, equipped with a 30 cm × 5.7 cm I.D. column, working with the same phase system.

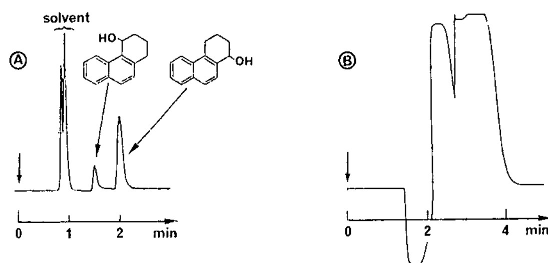

Fig. 1. Analytical (A) and small-scale preparative (B) separations of 1- and 4-hydroxy-1,2,3,4-tetrahydrophenanthrenes (4:1 synthetic mixture). Column: 30 cm × 0.42 cm I.D. Stationary phase, μPorasil, 10 μm. Mobile phase: dichloromethane–ethyl acetate (95:5). Detection: refractive index (RI). (A) Sample size, 100 μg in 10 μl. Flow-rate: 4 ml/min. Sensitivity: × 16. (B) Sample size: 54 mg in 1.75 ml. Flow-rate: 2.5 ml/min. Sensitivity: × 128 (from ref. 23 with permission).

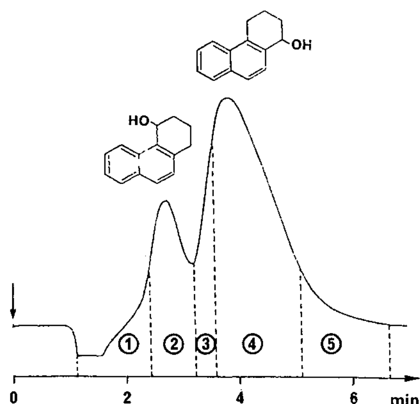

Fig. 2. Scale-up of the preparative separation of the tetrahydrophenanthrene mixture. Column: 30 cm × 5.7 cm I.D. Solvent system as in Fig. 1. Flow-rate: 300 ml/min. Particle size: 75 μm. Sample size: 10 g in 45 ml. Detection: RI (from ref. 23).

A preparative chromatogram, featuring fraction collection, is shown in Fig. 2. The 4-hydroxy isomer was recovered from fraction 2 in a yield of 85%, whereas the 1-hydroxy isomer was recovered from fractions 4 and 5 in a yield of 79%. The purities achieved were greater than 99%.

The analytical and preparative columns used in this study differed only in diameter and particle size. It is clear that the ratio of their volumes ($57^2/4.2^2 = 184.2$) was equal to the ratio of the sample sizes (10:0.054 = 185.2). Thus, the specific loads (defined as the sample to stationary phase ratio) were quite similar for the two columns.

To assess the pertinence of the operating conditions, let us first look into the working linearity of the analytical and preparative columns for the sample size considered as optimum. For the phase system used, the analytical capacity factors of both compounds were reported to be 0.63 and 1.38, which corresponds to a selectivity of 2.19. Assuming a total porosity of 0.80 for the column packing, the analytical retention volumes were calculated. The retention volumes corresponding to the optimal sample size, V'_{R_1} and V'_{R_2}, can be calculated from the flow-rate and the elution

TABLE II

RETENTION VOLUMES OF THE 1- AND 4-HYDROXYTETRAHYDROPHENANTHRENE ISOMERS FOR SMALL SIZE (V_R) AND OPTIMUM SIZE (V'_R) INJECTIONS ON THE ANALYTICAL AND PREPARATIVE COLUMNS

	Analytical column (30 cm × 4.2 mm I.D.)	*Preparative column (30 cm × 5.7 cm I.D.)*
V_{R_4} (ml)	5.4	1000
V_{R_1} (ml)	7.9	1455
V'_{R_4} (ml)	4.9	795
V'_{R_1} (ml)	7.0	1145
$[V_{R_4} - (V'_{R_4} - V_0/2)]V_{R_4}$ (%)	8	23
$[V_{R_1} - (V'_{R_1} - V_0/2)]/V_{R_1}$ (%)	11	23

time at the peak apex in Figs. 1B and 2. The results are given in Table II. By use of the linearity test, restricted to the comparison of the first statistical moments (retention volumes), a decrease in retention of *ca.* 10% for the analytical column is seen when it is used under optimum sample load conditions, indicating a slightly non-linear behaviour for this injection. The same treatment shows a more pronounced non-linear behaviour for the preparative column (23% decrease in retention) for a similar specific load. Assuming a packing density of 0.5 g/ml for silica, this sample load corresponds to about 26 mg of sample per gram of packing, which is very high. Thus, the observed non-linear behaviour is not surprising in this case. It might have been even more pronounced with larger sample molecules and higher capacity factors, In addition, at that high specific load there is no reason for using fine particles, for the major source of band spreading lies in the isotherm curvature[18].

Now, let us evaluate the optimum sample size suggested by the semi-empirical non-linear model allowing for mass overload. If we assume $C_m = 0.7\ M$ to allow for the small capacity factor of the more strongly retained isomer ($k'_2 = 1.38$) and $\tau_2 = 0.2\ V_{R_2} = 1.58$ ml, eqn. 2 gives the quantity to be injected as 43 mg for the more strongly retained isomer, *i.e.*, a sample of 54 mg for a 4:1 mixture. This is the quantity that was determined as optimal by the experimental approach.

Some other interesting conclusions may be drawn if we now consider the sample volumes and concentrations used for this separation. Systematic studies have shown that if the sample size which can be injected gives rise to pronounced non-linear behaviour, better resolution will be obtained with a small sample volume and high concentration[21]. To calculate the sample volume, the values of the analytical standard deviations, σ_1 and σ_2, of both peaks, in volume units, are needed. For the small-scale column, they can be evaluated by measuring the baseline intercept of the peak inflection tangents from Fig. 1A, by assuming gaussian peak shapes. Thus, $\sigma_1 = 0.16$ ml and $\sigma_2 = 0.22$ ml. Using these values and those of Table II, eqn. 1 enables one to calculate the maximum sample volume that can be injected in the case of a complete separation of both species under volume overload but without mass overload. A value of 1.73 ml is obtained. Thus, the volume actually injected into the analytical column (Fig. 1B) was obviously chosen so as to fit in with eqn. 1. However, the sample concentration, 0.16 *M* (31 mg/ml), greatly exceeds the highest known values for the upper limit of the linear range of the distribution isotherm. This choice of volume and concentration does not seem to be appropriate here, since the experimental results showed that mass overload can be contemplated. However, it is worth noting that, contrary to what is usually done when scaling up to a preparative column, the sample volume was increased by a factor much lower than the ratio of the column volumes. Taking into account the change in particle size from 10 to 75 μm between the two columns, it can be stated that the standard deviations of both analytical peaks on the preparative column are increased by a factor greater than the ratio of the column volumes. Thus, these standard deviations must be greater than 30 and 40 ml, respectively, and a sample volume of 45 ml like that actually injected into the preparative column did not contribute to bandspreading by volume-overload effects. Conversely, the sample concentration was increased to *ca.* 1.13 *M* (220 mg/ml) so as to keep the sample size proportional to the column volume. The increase in sample concentration explains why the non-linear behaviour of the preparative column is more pronounced than that of the small-scale column (Table II), in spite of quite

similar specific loads. Lastly, the linear velocity, which was very high for the analytical separation, was reduced to a medium value for the preparative column.

Finally, it can be said that the choice of the sample volume and concentration, which seemed questionable for the analytical column, appears to be perfectly adapted to a non-linear optimization strategy for the preparative column. In a case where this separation would have to be performed repeatedly, the flow-rate could be slightly increased to improve the production rate, since the flow-rate does not contribute much to the overall bandspreading under severe mass-overload conditions.

Stationary phase capacity limitation. Volume overload

Another significant example is the gram-scale separation of monensin A and B sodium salts described by Beran *et al.*[24]. These compounds are polyether antibiotics differing only by a methylene group (MW = 648 and 660) and were obtained from a fermentation broth. Their separation was carried out in the reversed-phase mode. An analytical chromatogram is presented in Fig. 3A. In order to determine the sample loadability of the chromatographic system, the sample size was progressively increased on the analytical column by varying the sample concentration and keeping the injected volume constant. For this study, the ratio, P, of the depth of the valley between the two peaks, f, to the mean peak height, g, was taken as the resolution criterion (Fig. 3B). Accordingly, the optimum sample size, determined as that corresponding to a P value of 0.9, was found to be 3 mg with a 500-μl sample volume. From these results, the separation was directly scaled up on a Jobin Yvon Chromatospac Prep 100 preparative chromatograph, using a 33.6 cm × 8 cm I.D. axially compressed column. The P values were measured as a function of sample size in the range from 0.85 to 2 g for a 25-ml sample volume. It was found that a 1-g sample size resulted in a P value of 0.8. Subsequently, this sample size was considered as optimum for this column (Fig. 3B). It

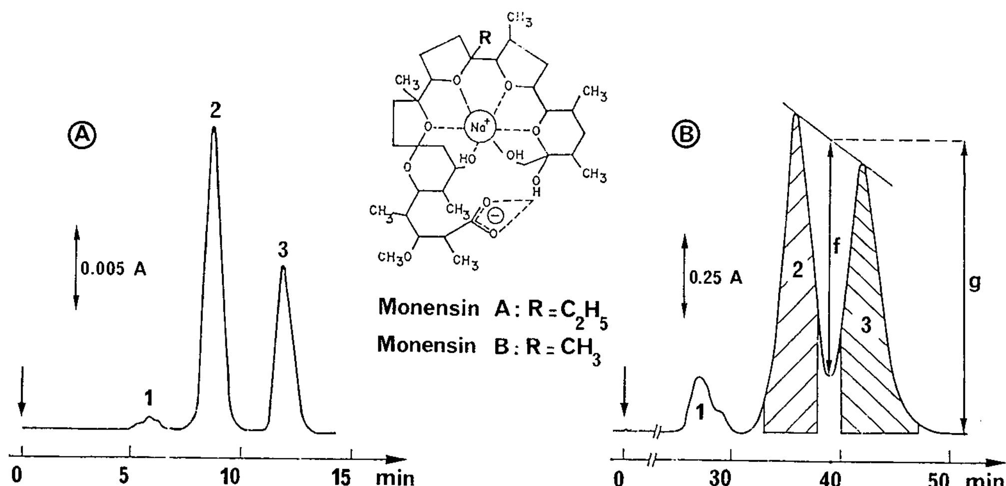

Fig. 3. Analytical (A) and preparative (B) separations of monensin A and B by reversed-phase chromatography on Separon C_{18} octadecyl silica with methanol–water (88:12). Detection: absorbance at 215 nm. Peak identification: 1 = unknown compounds; 2 = monensin B; 3 = monensin A. (A) Column: 34 cm × 0.4 cm I.D. Stationary phase particle size: 10 μm. Flow-rate: 1 ml/min. Sample size: 0.1 mg (500 μl). (B) Column: 33.6 cm × 8 cm I.D. Particle size: 15 μm. Flow-rate: 80 ml/min. Sample size: 1 g (25 ml) (from ref. 24 with permission).

TABLE III

CHROMATOGRAPHIC DATA FOR THE SEPARATION OF MONENSINS A AND B

	Analytical column (34 cm × 0.4 cm I.D.), 0.1 mg sample (500 μl) (measured)	*Preparative column (33.6 cm × 8 cm I.D.), 1 g sample (25 ml)*	
		Measured	*Calculated*
Retention volumes (ml)			
B	9.1	2855	3510
A	12.3	3375	4775
Standard deviations (ml)			
B	0.4		
A	0.4		
Hold-up volume* (ml)	2.4	944	
Total porosity	0.56	0.56	

* Given in the original paper[24].

allowed the recovery of 0.4 g of monensin B and 0.5 g of monensin A of 99.7 and 98.6% purity, respectively.

Using the data for the analytical column reported in Table III, a separation selectivity of 1.48 and an analytical resolution of about 2 can be calculated for the two compounds of interest. The chromatogram shown in Fig. 3A can be considered as the impulse response of the analytical column, in spite of the slight volume overload expected by comparing the injected volume with the measured standard deviations. The sample load regarded as optimal for that column (3 mg) was injected in a 500-μl volume of a *ca.* $9.3 \cdot 10^{-3}$ *M* mixture in the mobile phase. This concentration corresponds to the usual values of maximum concentrations, consistent with a linear distribution mechanism. However, eqn. 1 suggests that a volume of 1.6 ml can be injected without serious band overlapping due to volume overload. It would have been interesting to study the injection of *ca.* 4 mg mixture in 1.3–1.5 ml of a slightly more diluted solution, say about $5 \cdot 10^{-3}$ *M*.

If we now consider the scale-up of these injection conditions on the preparative column, it can be shown that the resolution parameter, P, decreases as a function of the specific load more rapidly on the preparative column than on the analytical one. This discrepancy cannot be ascribed to a difference in packing density, since both columns had the same total porosity (Table III), but rather to the fact that the sample load was increased by merely increasing the sample concentration at constant volume, while the ratio of the sample volume on both columns was lower than the ratio of the column volumes. Consequently, the preparative column exhibits a more pronounced non-linear behaviour than the analytical column for identical specific loads. Eventually, the optimum sample size and sample volume were increased by factors of $1000/3 = 333$ and $25/0.5 = 50$, respectively, whereas the column volume was increased by a factor of 395. Simultaneously, the sample concentration was raised to *ca.* $6.2 \cdot 10^{-2}$ *M* (40 mg/ml). This is probably above the maximum concentration allowed for linear distribution. Using the multiplicative factor approach[25], it also appears that, when scaling up the analytical conditions, the linear velocity was reduced by a factor of

$$\frac{F_{\text{anal}}}{F_{\text{prep}}}\left(\frac{d_{\text{c prep}}}{d_{\text{c anal}}}\right)^2 = 5$$

where d_c and F are the column diameters and flow-rates, respectively. This is expected to result in an improved resolution and a drastic decrease in pressure drop, as shown below, at the expense of separation time

$$R_{s\text{ prep}}/R_{s\text{ anal}} = \frac{1}{b}\sqrt{\frac{l}{u}} = 1.5$$

$$\Delta P_{\text{prep}}/\Delta P_{\text{anal}} = ul/b^2 = 0.088$$

where u, l and b are the ratios of the preparative- to analytical linear velocities, column lengths and particle sizes, respectively. Finally, it should be stressed that the measured values of retention volumes on the preparative column deviate from the calculated ones for the case of linear chromatography by about 19% for monensin B and 29% for monensin A, eluted later (Table III). This deviation, mainly due to the above-mentioned non-linear effects, appears to be a rather serious one for a sample size corresponding to a moderate specific load of about 1.2 mg sample per g of stationary phase (assuming a packing density of 0.5 g/ml). We think that this should be considered together with the low value of the column total porosity that can be calculated from the measured hold-up volumes (Table III). This might indicate a partial pore clogging and a decrease in specific surface area during the silica bonding process. The net result is, of course, a decrease in the available capacity of the stationary phase. A non-linear chromatographic behaviour more pronounced than expected was also observed by us in the case of the gram-scale enantiomer separation of a tertiary phosphine oxide on an aminopropyl silica, bonded with (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine moieties[26]. For a specific loading of only 2 mg/g, a decrease in retention volume by 32 and 35% was obtained with the two optical isomers, compared with their analytically determined values. This pronounced non-linear behaviour was related to the low number of chiral sites (34% of the aminopropyl sites, *i.e.*, 0.3 mmol/g) available on the stationary phase, resulting in a low capacity.

In the separation of monensins, with this phase system, the preparative column can certainly accommodate a much larger sample volume than that actually injected. This would allow reduction of the sample concentration and thereby, non-linear effects. Another approach might be to use a bonded phase having a more common value of the total porosity, in order to improve column loadability.

Sample volume; column packing; injection device

Another large-scale separation of interest is that of an equimolar mixture of two menthyl methylphenylphosphinate diastereoisomers, performed in our research group[27]. An appropriate phase system was first investigated on a 20 cm × 0.48 cm I.D. analytical column. The best conditions are given in Fig. 4A. The separation was next scaled up on a 70 cm × 1.0 cm I.D. column, packed with the same silica gel and operated with the same mobile phase. A sample size of 30 mg, corresponding to

a specific load of 1 mg/g, was first injected by use of a syringe. The resulting chromatogram is shown in Fig. 4B. Still using a syringe, the sample size was then increased to 90 mg (0.3 ml of a 300 mg/ml solution in the mobile phase). For such a load, the purities of the recovered isomers were 95 and 97%, respectively, in a yield of 88%. Twelve identical injections of this size were performed without waiting for the elution of both isomers between injections (short cycling technique), so that 0.48 and 0.47 g, respectively, of each isomer were obtained in only 5 h.

The data obtained from a small-size injection on the analytical column (Fig. 4A) and from a 30-mg injection on the preparative column (Fig. 4B) are given in Table IV. Assuming a total porosity of 0.75 for these columns, we can calculate a selectivity of 1.12 from the analytical results, which indicates a rather difficult separation. The resolution calculated from the values of retention volumes and standard deviations was only 1.03. This resolution was improved by choosing a preparative column longer than the analytical one. The chromatographic characteristics of a small-size injection into the preparative column, which were not given in the original paper, were calculated, assuming that, at constant linear velocity, the retention volumes are proportional to the column volume, and the plate number and pressure drop are proportional to the column length. Thus, a resolution close to 1.9 is expected on the preparative column.

However, it appears that the retention volumes of both compounds on the preparative column for a 30-mg sample injection (0.1 ml) are 23–24% higher than those predicted on this column for a small size sample (Table IV). This increase in retention volume cannot be ascribed to a volume- or mass-overload effect. An unexpected change in solvent composition may be responsible, but this seems very doubtful. A more likely explanation would be that the analytical and preparative columns do not have identical total porosities, the porosity of the preparative column

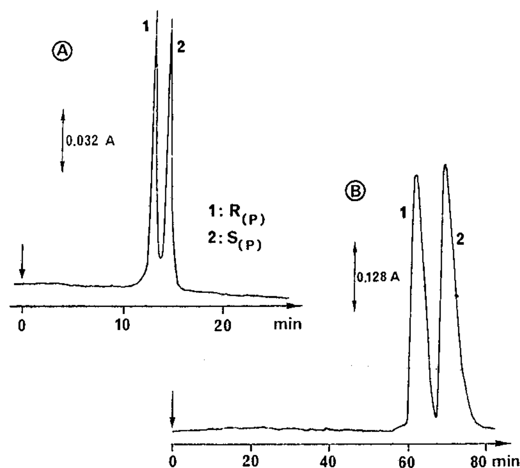

Fig. 4. Analytical and preparative separation of two menthylphenylphosphinate diastereoisomers. Stationary phase: Partisil 5 μm. Mobile phase: hexane–methanol (98.5:1.5), water content 0.01%. (A) Column: 20 cm × 0.48 cm I.D. Flow-rate: 100 ml/h. Pressure drop: 40 bar. Sample: 5 μl of a 10 mg/ml solution. (B) Column: 70 cm × 1.0 cm I.D. Flow-rate: 400 ml/h. Pressure drop: 110 bar. Sample: 100 μl of a 300 mg/ml solution (from ref. 27 with permission).

being higher than that of the analytical one. This explanation is consistent with the value of the ratio of the preparative to analytical column permeability, 1.18, which can be calculated from the pressure drop measurement given in Table IV. These remarks suggest that the preparative column was not packed densely enough. This is not surprising for a column 70 cm in length, packed by conventional methods. Likewise, the standard deviations, measured for a 30-mg injection, are higher than the predicted values for a small-size injection (Table IV) and their ratios are similar for the two compounds. This discrepancy can be attributed to less efficient packing of the preparative column rather than to extra-column or non-linear elution band broadening.

Using eqn. 1, the maximum sample volume that can be injected in the case of linear behaviour of the column was found to be about 17.6 ml. The corresponding maximum solute concentration was estimated to be of the order of $7\text{–}8 \cdot 10^{-3}$ M (strongly retained solutes having capacity factors of 7.1 and 8). Thus, using the linear approach, the optimum sample size of the isomer mixture (MW = 294) would be *ca.* 75 mg. If we take into account non-linear distribution effects, the maximum sample size will amount to about 370 mg. For this last calculation, 0.5 M and 74 ml = 0.2 V_{R_2} were taken for $C_{m,2}$ and τ_2, respectively, and substituted into eqn. 2. As expected from theory, this sample size should be injected as a concentrated solution, *e.g.*, 1.25 ml of a 300 mg/l solution.

If we now consider the actual experimental results, we can see that the sample size considered optimal from a practical point of view is close to that calculated for linear elution, whereas the small sample volume and the high concentration would correspond to a non-linear elution separation. At this point, one wonders why the sample volume actually injected was so small. It is likely that it is impossible to inject a larger volume by means of a manual syringe without detrimental effect on band broadening or simply because of the pressure resistance of the column packing.

From the foregoing discussion it may be concluded that, in order to increase the sample througput of this separation, further investigations are needed in two directions: first, to obtain a more efficient preparative column with a denser bed by a more appropriate packing method, *e.g.*, a method involving axial and/or radial

TABLE IV

CHROMATOGRAPHIC CHARACTERISTICS OF THE SEPARATION OF THE MENTHYL PHOSPHINATE ISOMERS ON THE ANALYTICAL AND PREPARATIVE COLUMNS

V_R = Retention volume; σ = standard deviation.

	20 cm × 0.48 cm I.D. column, small-size sample (measured)	*70 cm × 1.0 cm I.D. column*	
		Small-size sample (calculated)	*Sample: 0.1 ml of a 300 mg/ml solution (measured)*
V_{R_1} (ml)	22.0	333	415
V_{R_2}(ml)	24.4	370	465
σ_1 (ml)	0.52	4.2	10.6
σ_2 (ml)	0.64	5.2	12.4
Pressure drop (bar)	40	140	110
Flow-rate (ml/h)	100	433	400

compresion; secondly to make use of a sample loop valve together with a device affording an even sample distribution over the column cross-section at the column inlet.

Mass overload; sample introduction

The last example chosen to illustrate this methodology is a separation of 1- and 4-diamentanol isomers, reported by Kříž *et al.*[28] on a 27 cm × 4 cm I.D. axially compressed column, packed with 10–20 μm silica gel (Fig. 5). Because these compounds were poorly soluble in potential mobile phases, a method of solid sample introduction was used. A silica layer of about 1-cm thickness was removed from the top of the column and mixed with approximately half the amount of solid sample before being packed into the column. This was easy to do with axially compressed columns, although difficult to automate, and it was shown to provide higher purities and recovery yields for sample sizes of the order of 2–3 g. However, for a sample size of 5 g, the elution pattern displayed long tails as evidenced by gas–liquid chromatographic analysis of small fractions. The authors wondered about the contributions of isotherm non-linearity and of solid-sample introduction to this peak tailing, which prevented them from recovering any pure fraction, of the later-eluted isomer. To answer this question, it is worth noting that the tails of both peaks extended far beyond the retentions corresponding to $V_R + 3\sigma$ (V_R and σ being the retention volume and standard deviation, respectively, determined under analytical conditions), as if there

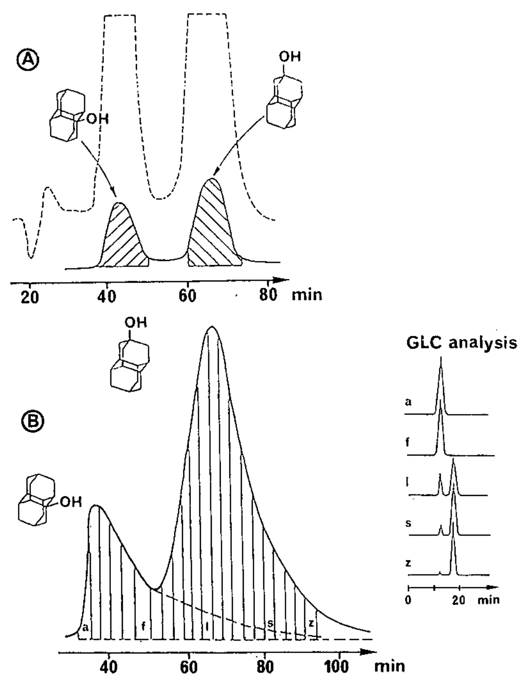

Fig. 5. Preparative separation of 1- and 4-diamantanol isomers. Column: 27 × 4 cm I.D. Stationary phase: 10–20 μm silica gel. Mobile phase: *n*-pentane–2-propanol (98:2). Flow-rate: 22 ml/min. Sample size introduced in solid state: (A) 1 g; (B) 5 g. RI detection (from ref. 28 with permission).

were a large volume overload. Although the specific load was high in that case (25 mg/g), such a phenomenon is not consistent with the known peak shapes obtained with non-linear, convex adsorption isotherms[11,16–18,29]. To our mind, it can be explained only by a slow dissolution of the sample in the mobile phase. It seems likely that, beyond a certain amount of sample introduced in the solid state, peak tailing is controlled by the rate at which the sample dissolves. Furthermore, an amount of silica equal to twice the sample amount seems inadequate to accommodate all of the sample. Based on literature capacity data[16,30], the amount of silica should be *ca.* 4 to 5 times greater than the sample amount.

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CHROMSYMP. 1398

COLUMN LOADING AND RELATIVE RETENTION IN OVERLOADED ELUTION CHROMATOGRAPHY

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SUMMARY

The influence of the relative retention, α, on the column loading capacity was investigated for compounds having slowly diverging Langmuir isotherms. The same trends as observed previously for a binary mixture with $\alpha = 1.09$ were found. Larger sample sizes were required in order to observe band overlap when α increased. Accordingly, the displacement and tag-along effects observed on the band profiles were stronger. In all instances, the recovery decreased with increasing sample size. The production rates, on the other hand, increased at first, passed through a maximum and then decreased. It is also shown that the optimal production increases with increasing α.

INTRODUCTION

Preparative-scale liquid chromatography is rapidly becoming a major purification technique as the need for large amounts of highly pure compounds has increased dramatically in the last few years. This renewed interest has followed the tremendous growth of the biotechnological and pharmaceutical industries.

When dealing with chromatographic separations, it is important to optimize the resolution, separation speed and sample loading capacity[1]. As these parameters are interrelated, a compromise must be sought. In analytical chromatography, for example, data collection and analysis are emphasized. Accordingly, the separation speed and/or resolution are maximized at the expense of sample loading. In preparative chromatography, on the other hand, the goal is a higher sample capacity and the separation speed and/or resolution are therefore of secondary concern.

The most important factor influencing these characteristics in analytical chromatography is the relative retention, α, which is the ratio between the column capacity factors of the two compounds to be separated. The resolution, R_s, for a very small sample size is given by the classical equation[2]

$$R_s = \frac{1}{4}\sqrt{N}(\alpha - 1)\left(\frac{k'}{1 + k'}\right) \tag{1}$$

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where N is the column efficiency and k' is the column capacity of the second component of the pair. For a given binary mixture, the larger the α value, the better is the analytical resolution and the faster the analysis. Therefore, chromatographic systems are selected in order to maximize the relative retention of the pairs of compounds that are most difficult to separate.

Intuitively, we may conclude that the column loading capacity will also be maximized by the selection of the chromatographic system that affords the greatest α value. This is true if the binary equilibrium isotherms of each of the two compounds are slowly divergent, as seems to be the general case for similar compounds and especially for closely related isomers. This may not be so for compounds that would undergo strong sorbate–sorbate interactions in the stationary phase or for compounds for which the column loading capacities would be very different. If the column capacity is much larger for the lesser retained compound than it is for the more strongly retained compound then the binary isotherms may cross each other, resulting in a practical limit to column overloading which may be abnormally low and lead to paradoxical results at larger loads. This paper deals with what we feel is the general case. A forthcoming paper will discuss an example of the latter case.

THEORETICAL

The semi-ideal model

We calculated elution profiles for large samples of binary mixtures using the previously described and discussed semi-ideal model[3,4]. In this model, a mass balance equation is written for each component of the mixture. It is assumed that the mobile phase is not adsorbed, which is acceptable for a one-component mobile phase and depends on the reference state chosen for adsorption[5]. For binary or more complex mobile phases, the assumption still holds, but only for the weak solvent. Depending on the experimental conditions, dropping the strong solvent mass balance equation may still lead in many instances to correct predictions of the elution band(s) of the solute(s), but complications will arise as it will no longer be possible to account for ghost (or system) peaks.

The system of mass balance equations should be completed by a relationship between the concentrations of each solute in the mobile and stationary phases. This should be given by a kinetic equation written for each component of the binary mixture. In the ideal model of chromatography, it is assumed that the kinetics of mass transfer are so fast that equilibrium between phases is reached instantaneously, so the column has an infinite efficiency. The relationship between the solute concentrations in the mobile and stationary phases is then given by the competitive equilibrium adsorption isotherms. This corresponds to the well studied ideal model of chromatography[6–9]. Finally, the calculations of numerical solutions of the ideal model require a set of boundary conditions describing the injection.

The inherent limitations of the ideal model lie in the appearance of concentration shocks on the elution profiles. The appearance of these shocks and their stability result from the absence of a diffusion term in the partial differential equations. This is a consequence of assuming an infinite column efficiency and does not lead to very realistic results. Further, during the millions of calculation loops, the computer must first locate these shocks. It then calculates the concentration values on both sides of

the discontinuities and finally it extrapolates between these two values. This procedure leads to an unacceptable loss of peak area (*i.e.*, matter) during the calculation[9].

In this work, we used a finite difference method based on the Godunov algorithm[10]. The continuous (z,t) plane is replaced by a $(n\delta z, i\delta t)$ grid defined by a space and a time increment. The space increment, δz, is set equal to the height equivalent to a theoretical plate of the column, H, and the time increment, δt, to twice the time required for a non-retained compound to move a distance equal to $2H$ down the column. It can be shown that with these increment values, the errors resulting from the replacement of the partial differential equations by finite difference equations mimic exactly the effect of a finite column efficiency on the elution profiles[11]. The numerical errors introduced are equivalent to a diffusion term where the diffusion coefficient is equal to the apparent diffusion coefficient of the chromatographic column used. The only assumptions made are that the mass transfer coefficients (such as the molecular diffusion coefficients) are independent of the concentration of solute, which is true in the range used in preparative liquid chromatography, and that H does not depend on the retention time (*i.e.*, k'). Accordingly, the concentration shocks do not appear, the profiles are realistic and there is no loss of matter during the computer calculations.

The simulation

A FORTRAN program allows the calculation of numerical solutions of the system of partial differential equations. It permits the elution profiles of both components of the mixture to be obtained for a well defined set of experimental conditions, provided that the competitive equilibrium adsorption isotherms are known. Eventually, by integrating the elution profiles, the recovery yields and production per unit time for each component of the mixture can be determined[3].

For a two-component mixture, the difficulty of the separation depends on the relative retention, α, of the two compounds and on the ratio of the isotherm curvatures. In this paper, we have assumed that the ratios of the slopes and of the curvatures of the two binary isotherms are equal. This corresponds to two binary isotherms which are slowly diverging. While the precise combination of numbers is arbitrary, the situation seems to be fairly general for pairs of closely related compounds. For others, a change in the chromatographic system will most often transform a separation that is accidentally difficult into an easy one. We have investigated the effect of the relative retention on preparative separations for different relative concentration ratios (1:9, 1:3 and 9:1).

In liquid chromatography, many binary mixtures have adsorption isotherms that can be described reasonably well, to a first approximation, by competitive Langmuir adsorption isotherms of the form

$$q_i = \frac{a_i c_i}{1 + b_1 c_1 + b_2 c_2} \tag{2}$$

where q_i and c_i are the concentrations of component i in the stationary and mobile phases, respectively; a_i and b_i are the Langmuir parameters for component i, a_i being given by the equation

$$a_i = k'_i \cdot \frac{V_m}{V_s} \tag{3}$$

where k'_i is the column capacity factor of component i for a very small sample size and V_m and V_s are the mobile and stationary phase volumes, respectively.

The column is defined by its total porosity, which is set equal to 0.8, a value frequently encountered in liquid chromatographic columns. Hence,

$$\frac{V_m}{V_s} = \frac{\varepsilon}{1 - \varepsilon} = 4 \quad (4)$$

In all instances, the column length is 25 cm while its efficiency, as defined by the number of theoretical plates N, is equal to 5580 for a very small sample size. As shown previously[3,4,6–11], the broadening of the peak with increasing sample size observed experimentally is based on the thermodynamics of the process (non-linearity of the equilibrium adsorption isotherm). It does not reflect a change in the kinetics of mass transfer between phases, *i.e.*, it cannot be explained by a decrease in the actual column efficiency. The characteristics k', a and b of the more strongly adsorbed component of the mixture (2) are kept constant while those of the lesser retained compound (1) are changed. Computer simulation is used to study the influence of the relative retention on the preparative separations of binary mixtures whose competitive Langmuir isotherms are known. The numerical values introduced into eqn. 2 for each solute are reported in Tables I and II. The k'_i values listed correspond to the injection of a very small sample size. The retention time of an unretained solute is 40 s.

The sample size is given in arbitrary units. The column saturation capacity, as defined by Eble *et al.*[12], is equal to 100 so that a sample size of 10 units corresponds to a 10% column capacity loading.

TABLE I

NUMERICAL VALUES USED IN EQN. 2 FOR $\alpha = 1.25$

Component	k'	a	b
1	5	20	2.07
2	6.25	25	2.56

TABLE II

NUMERICAL VALUES USED IN EQN. 2 FOR $\alpha = 1.7$

Component	k'	a	b
1	3.675	14.7	1.52
2	6.25	25	2.56

RESULTS AND DISCUSSION

Influence of the relative retention on the preparative separation of a 1:3 mixture

For $\alpha = 1.25$, a 5-unit sample size of the mixture (5% of the column saturation capacity) is sufficient to allow for some interaction between the two bands (Fig. 1a). With a 20-unit sample size, there is a considerable interaction between the two bands (Fig. 1b). The concentration discontinuities on both chromatograms are extremely sharp, even with the smoothing effect of diffusion; the shock layer (*i.e.*, the region where the concentrations of both components 1 and 2 vary rapidly) has become thin[13]. Compound 1 is concentrated in a narrow band in front of the second band. This phenomenon increases with increasing sample size (see Fig. 1a and b), as indicated by the increase in the peak maximum of component 1 from approximately 0.78 to 2.8. The lower part of the diffuse side of the first peak, however, continues to drag behind, underneath the second peak profile well after the more strongly adsorbed compound has started to elute.

The front of the peak of compound 2 elutes faster than if it were alone and a hump forms on its tail that becomes larger with increasing sample size.

What happens here is that, at the beginning of the column, the concentration of component 2 is very large and its molecules occupy most of the sites on the stationary phase. As a result, component 1 is pushed in the mobile phase and moves faster than if it were alone in the column. This signals the onset of a displacement effect[3,14]. After the peak maximum of component 2 has been eluted, however, its concentration decreases, thus freeing some sites on the stationary phase on which component 1 can be adsorbed. Component 2 then competes less strongly with 1 which, in turn, is less strongly displaced, *i.e.*, it lags behind and an isotachic train can never form. This phenomenon is characterized by the strong tailing of the lower part of the diffuse rear of the peak of component 1.

These phenomena are dramatically illustrated by a comparison of Fig. 2a and b, which correspond to a larger α value of 1.7. Fig. 2a shows that for a 15-unit sample size (15% of the column saturation capacity) completely resolved (albeit overloaded) peaks are eluted. This is the best way to achieve complete recovery of both components of the mixture, as illustrated by Knox and Pyper[15].

A comparison of Figs. 1a, 1b and 2a confirms that the loading capacity of a column increases with increasing α. Fig. 2b shows the superimposition of the chromatograms obtained successively for pure components 1 and 2, in amounts equal to those used for Fig. 2a (3.75 units of 1, then 11.25 units of 2). It can be seen that the profiles of component 2 injected alone and in the mixture are identical. This demonstrates the stability of the band profile. Its shape, which is imposed by solution thermodynamics and by the dynamics of band migration in non-linear chromatography, is restored after perturbation of the frontal shock. Compound 1, however, is displaced from a retention time of 134 s (pure) to a time of 111 s (mixture). Its band width narrows from 57 to 37 s and its maximum concentration increases from 1.6 to 2.1 m*M*. This phenomenon, which has been called the "blockage" effect[12], appears to be the residual consequence of the displacement effect which takes place earlier in the column.

The chromatogram in Fig. 2a is very similar to that corresponding to the reversed-phase separation of two xanthines [β-hydroxyethyl- and 7-β-hydroxypro-

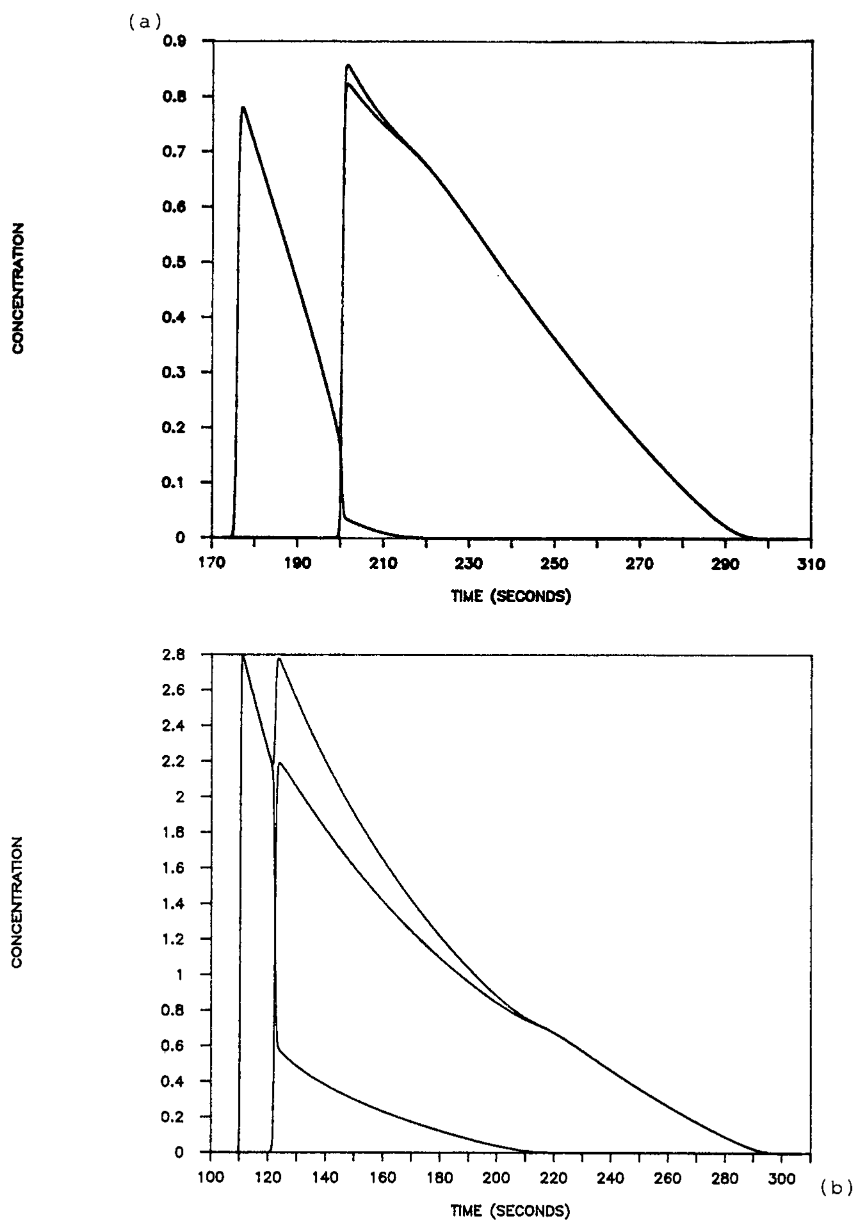

Fig. 1. Overloaded chromatograms of a 1:3 mixture with α = 1.25. (a) Sample size 5% of column capacity; (b) sample size 20% of column capacity.

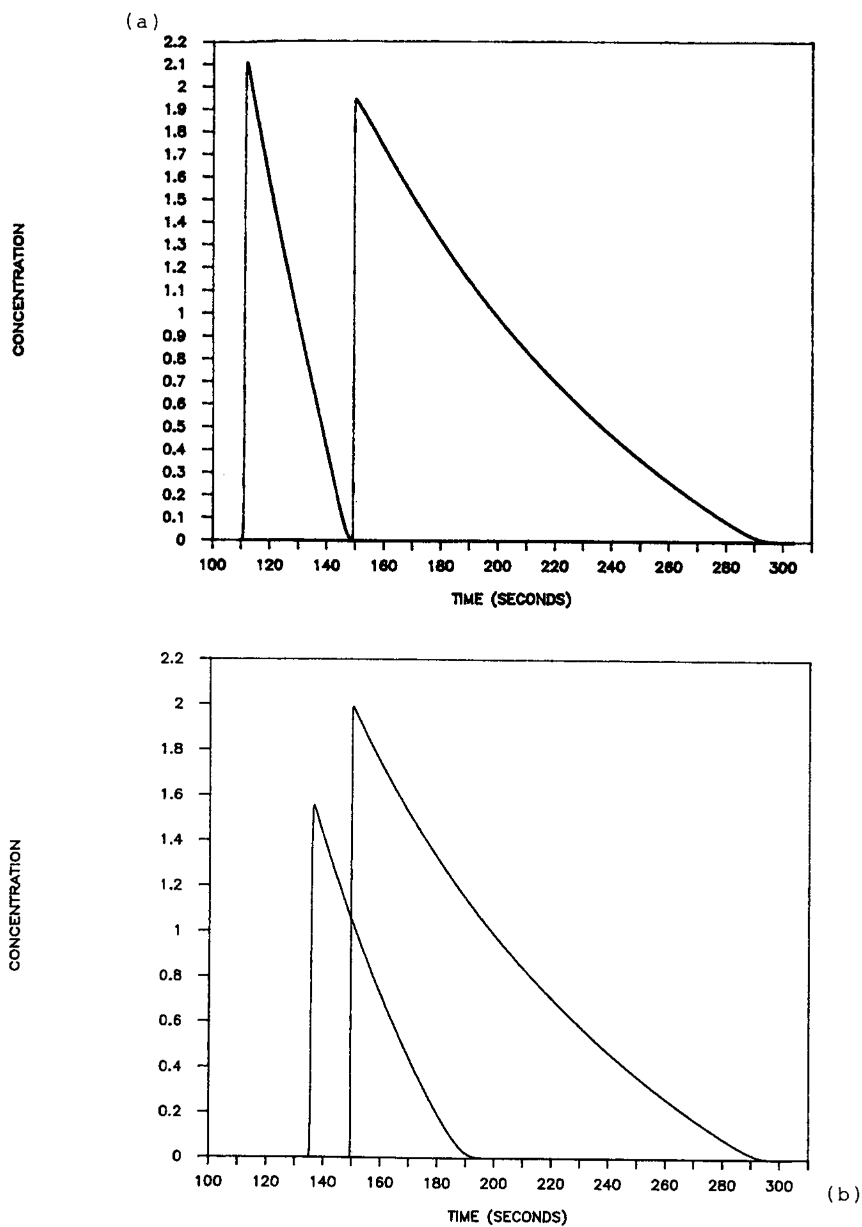

Fig. 2. Overloaded chromatograms of a 1:3 mixture with α = 1.7 and a sample size equivalent to 15% of the column capacity (column length 25 cm). (a) 1:3 mixture of compounds 1 and 2; (b) same amounts of compounds 1 (3.75% of the column capacity) and 2 (11.25%), injected successively.

pyltheophylline (HET and HPT)] by Eble *et al.*[16] (see Fig. 4 in ref. 16). The main difference resides in the retention times; in our simulation, compound 1 is less retained and is therefore narrower, and hence more concentrated than in the experimental case. It should be emphasized that although the two bands are completely resolved at the column outlet, they interact very strongly in the column, especially at the beginning of elution. The displacement effect decreases slowly in importance as the bands migrate along the column and slowly become disengaged from one another (see Fig. 3a and b). This effect is more pronounced for smaller values of α as they correspond to compounds whose affinities for the stationary phase are more comparable. Also, the tag-along effect of the second band decreases with increasing migration distance. As the two bands separate, the front of the second band recedes relatively, the profile of this second band becoming progressively more identical with that of a pure sample of compound 2. This is because the velocity of the self-sharpening front of a pure compound band depends only on the concentration of the band maximum and decreases with decreasing maximum concentration. The tag-along effect pulls forward the front of peak 2, decreasing its maximum concentration (see Fig. 2). When the bands separate, the tag-along effect decreases, the front of band 2 recedes and its maximum height increases. Thus, the band returns to the stable profile of pure compound 2 (Fig. 2b).

Fig. 4a and b shows plots of the recoveries for component 1 *versus* amount injected, for α = 1.25 and 1.7, respectively. As expected, the yields decrease much faster with increasing sample size for α = 1.25 (*e.g.*, 60% compared with 98% for 20% column saturation capacity). At 50% of the column saturation capacity, the recovery of 1 is still greater than 70% for α = 1.7. The same general trends are observed for the second component of the mixture. Clearly, a large α value allows for a greater loading capacity. Hence it is certainly good practice to optimize first the analytical resolution before undertaking preparative high-performance liquid chromatography (HPLC). A larger throughput of the mixture of interest will be achieved, provided that the ratio of the curvatures of the isotherms at the origin is larger than 1.

Fig. 5a (α = 1.25) and b (α = 1.7) shows the production per unit time of component 2 as a function of the total amount of the mixture whose introduction into the column is simulated. In all instances the optimal production shifts towards a higher loading capacity when the required sample purity decreases. This can be understood in terms of the strong tailing of component 1 underneath 2. It is a source of contamination and reduces its yield markedly. In practical terms, the less stringent are the purity requirements, the more overlap, *i.e.*, interactions, can be allowed between the two components of the mixture. Finally, the higher the α value, the greater is the possible throughput and the higher is the optimal production at a given required purity.

Influence of the relative concentration of the components of a binary mixture on their preparative separation for α = 1.7

We illustrate the influence of the relative concentration with the chromatograms obtained in two extreme cases, corresponding to relative concentrations of 1:9 and 9:1.

Fig. 6a and b shows the chromatograms for a 1:9 mixture, with sample sizes

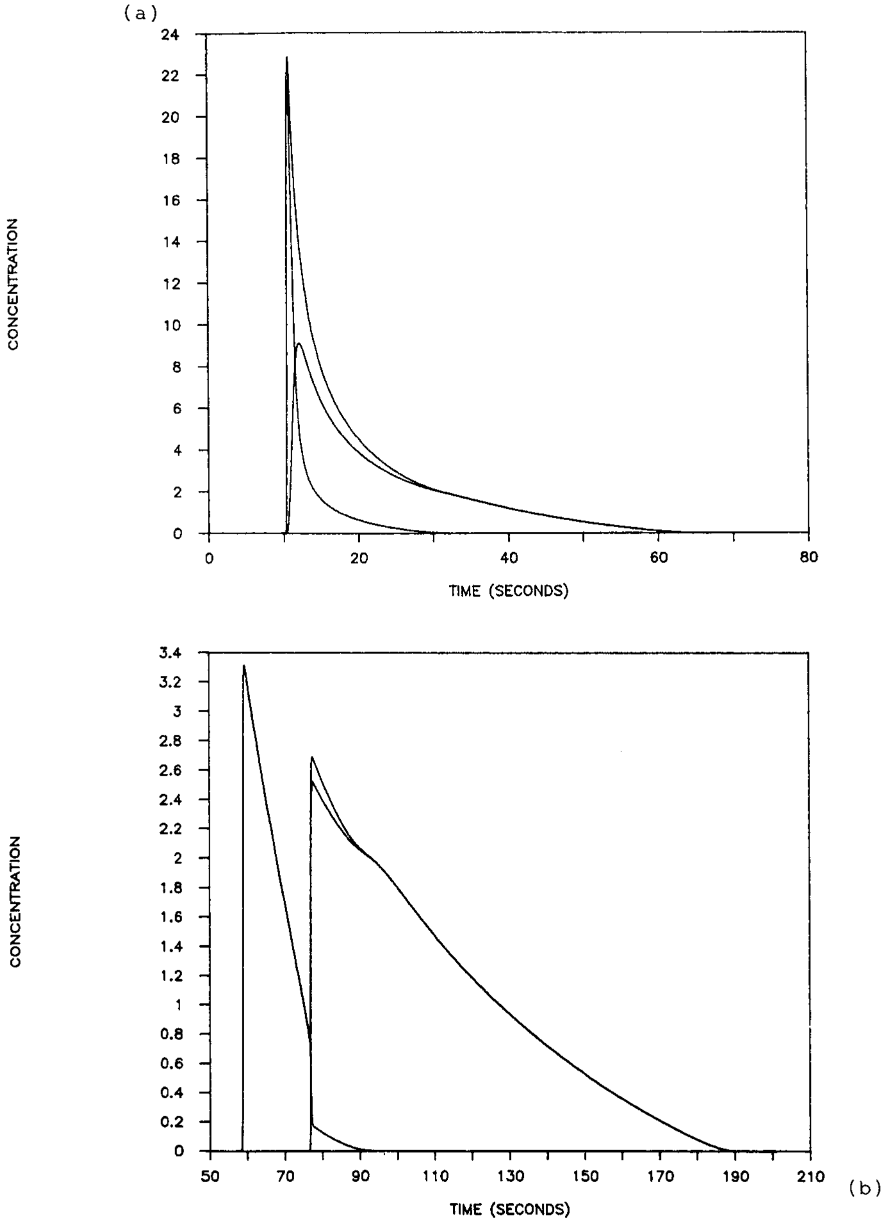

Fig. 3. As Fig. 2a but chromatograms recorded at different column lengths: (a) z = 5 cm; (b) z = 15 cm.

equal to 20 and 50% of the column capacity, respectively. In Fig. 6a the two bands interact only slightly, whereas they interact very strongly in Fig. 6b. In both instances component 2 is in large excess and it therefore displaces 1 in front of it and concentrates it into a thin zone. This phenomenon is illustrated by the narrowing of the band of 1 with increasing sample size and the increase in the concentration corre-

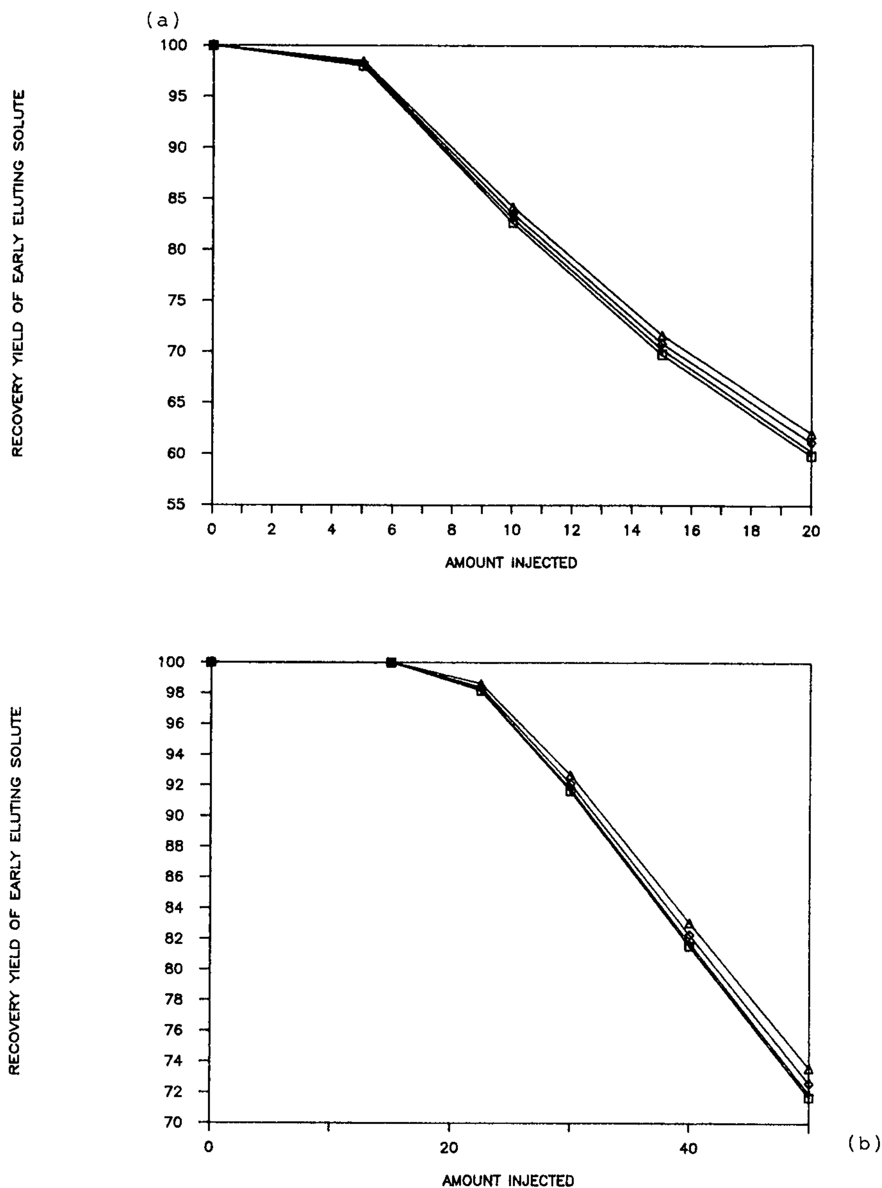

Fig. 4. Plot of recovery of compound 1 *versus* total amount injected (1:3 mixture): (a) α = 1.25; (b) α = 1.7. Purity of the recovered fractions = 99% (□); 95% (◇); 98% (+); 90% (△).

sponding to its peak maximum from approximately 1.9 to 8.3. Moreover, the displacement effect is emphasized by the fact that although the yield of 1 decreased from 98% to 70% (Fig. 7a) when the sample size increased from 20 to 50% of the column capacity, its production per unit time (Fig. 7b) increased by more than 33% from

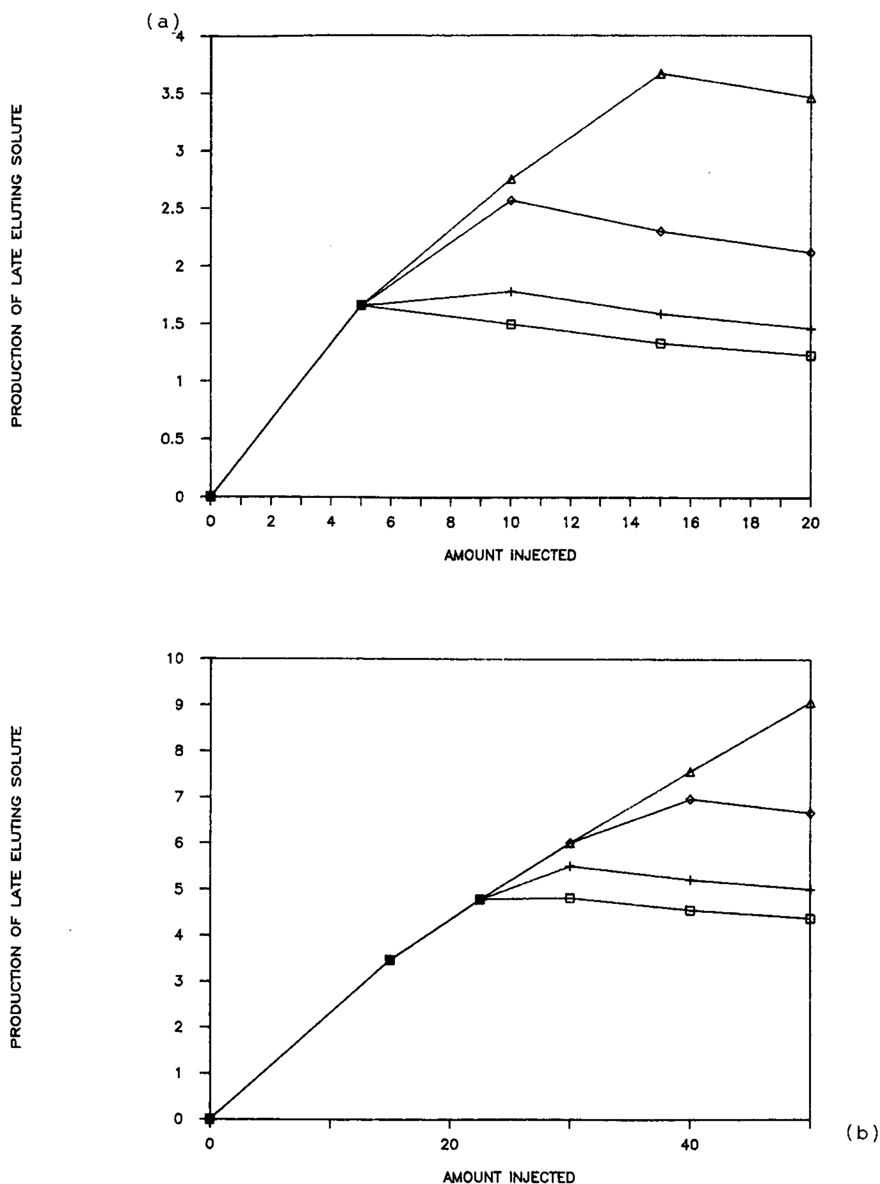

Fig. 5. Plot of production rate of compound 2 *versus* total amount injected (1:3 mixture): (a) α = 1.25; (b) α = 1.7. Key as in Fig. 4.

less than 0.6 to 0.8. Fig. 7b clearly indicates that a column loading equivalent to 50% of the column capacity leads to an optimal production per unit time for the lesser retained compound.

Finally, we note that the production and recovery of compound 1 depend very little on the required purity (see Fig. 7a and b). In contrast, the production and

recovery of component 2 depend very much on the required purity of the product. Fig. 8a shows a recovery of almost 100% for a required purity of 90% at 80% of the column saturation capacity compared with 30% for 99% purity. This can be traced back to the tail of 1 behind the steep part of the tail, at the border separating the bands of 1 and 2 (see Fig. 7a and b). The production of component 2 increases with increasing throughput until a very weak maximum is observed (Fig. 8b). The optimal throughput shifts to higher loading capacity as the required purity decreases. Again, it should be noted that the optimal production always occurs far after the two bands have merged together.

The chromatograms obtained for a 9:1 mixture are very different. For a sample corresponding to 20% of the column capacity, the bands of compounds 1 and 2 are completely resolved. There is hardly any difference between the profiles obtained for component 1 with this mixture or with a pure sample, and a size corresponding to 18% of the column capacity (same amount of 1). No displacement effect is observed, as illustrated by the lack of a sharp front between the profiles of the two compounds (Fig. 9a). The band of 2, in contrast, has a profile that is very different from the profile of a pure compound band. It exhibits a strong "tag-along" effect[12], *i.e.*, the lesser retained compound 1 drags component 2 forward. Peak 2 is very shallow and presents a much larger band width than when injected alone on the column. As the column loading is increased compound 2 interacts increasingly with 1, as these effects are non-linear, and its band is therefore dragged forward more and more. As a result, its band becomes shallower to the point of becoming unnoticeable, *i.e.*, undetected as seen in Fig. 9b. For a 50% column capacity sample of a 9:1 mixture, the ratio of the maximum height of band 1 to the nearly constant height of band 2 is about 50. To the chemist unaware of non-linear effects, it might seem that compound 2 has disappeared. Nevertheless, it is striking that the recovery (Fig. 10a) is still more than 50%. For an injection corresponding to 80% of the column saturation capacity, more than 30% of 99% pure 2 can still be recovered (Fig. 10a). This is because, with the large difference between the adsorption isotherms of compounds 1 and 2, compound 2 never interacts with 1 (Fig. 9b). Fig. 10b further indicates a levelling off of the production rate of 2 between 30 and 80% of the column capacity. In this specific instance, a 30% column loading would be advisable. It corresponds to the optimal production and a still high recovery (80% for P = 90%, 72% for P = 99%).

It might seem surprising to observe (Fig. 9a) the two compounds being completely resolved, the band profile of compound 1 being nearly identical with that obtained with a pure compound sample and the band profile of 2 so considerably broadened. This is, however, merely the converse of the displacement effect observed in Fig. 1a. The bands of the two components interact considerably at the beginning of the separation process, in the first part of the column, as they are not resolved until they elute (see Fig. 3a and b). In Fig. 1a, the major effect is displacement and band 1 is moved forward and compressed. In Fig. 9a, the tag-along effect is predominant and band 2 is moved forward and spread. In this instance, there is a marked dilution of compound 2 in the process. It decreases the extent of non-linear phenomena and prevents the band profile from recovering and returning to the classical single component profile. The opposite is true when the displacement effect is predominant (see Fig. 1a).

Finally, we have simulated chromatograms corresponding to the separation of

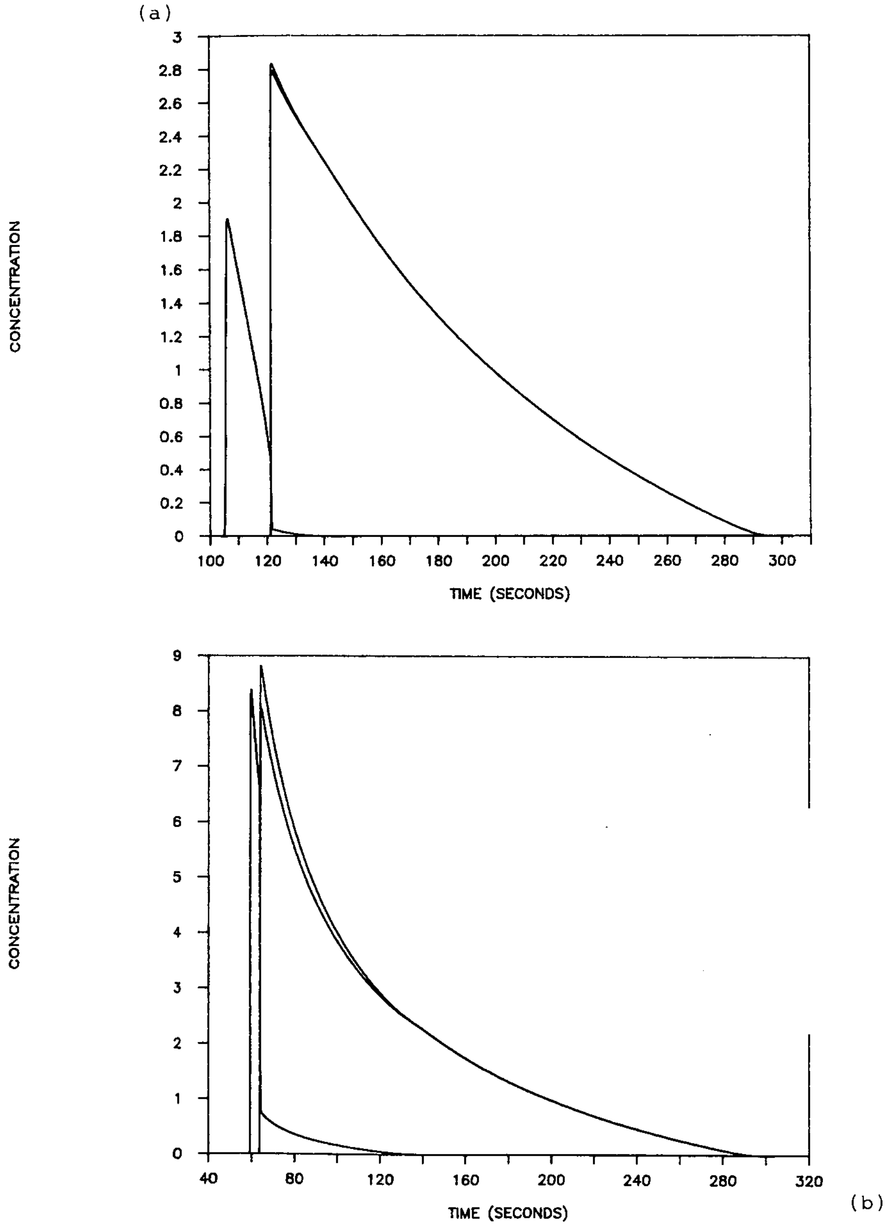

Fig. 6. Overloaded chromatogram of a 1:9 mixture and a sample size corresponding to (a) 20% of the column capacity and (b) 50% of the column capacity.

similar mixtures with a relative retention of 3.0. The results obtained exhibit the same phenomena, with stronger non-linear effects of displacement and tag-along effects due to the large sample size needed to achieve band interaction.

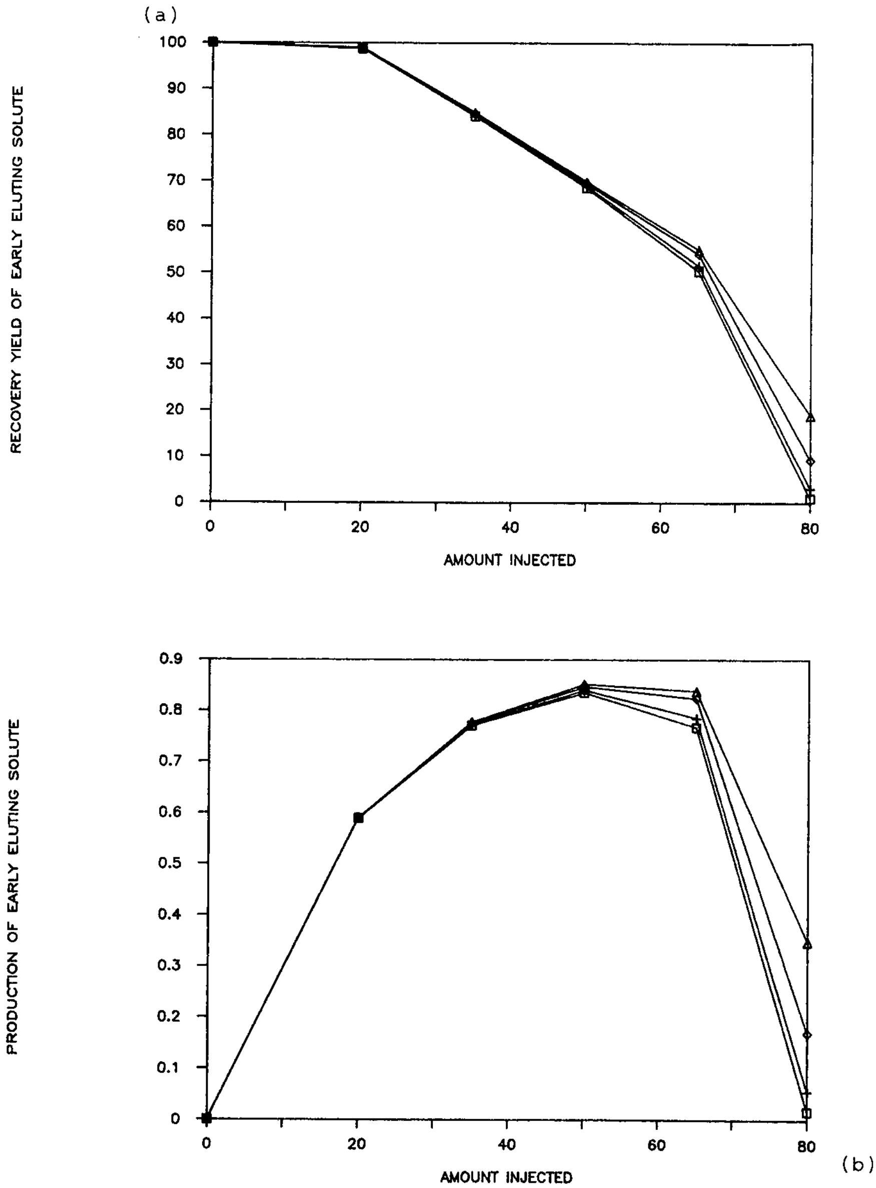

Fig. 7. Component 1 of a 1:9 mixture (α = 1.70): (a) plot of recovery *versus* sample size; (b) plot of production rate *versus* sample size. Key as in Fig. 4.

CONCLUSION

Comparison of Figs. 1 and 2, 4a and 4b and 5a and 5b and with the results of our previous work[3,5,14] shows that the same non-linear efffects take place during the separation of a binary mixture whether the relative retention is 1.09, 1.25, 1.7 or 3.0.

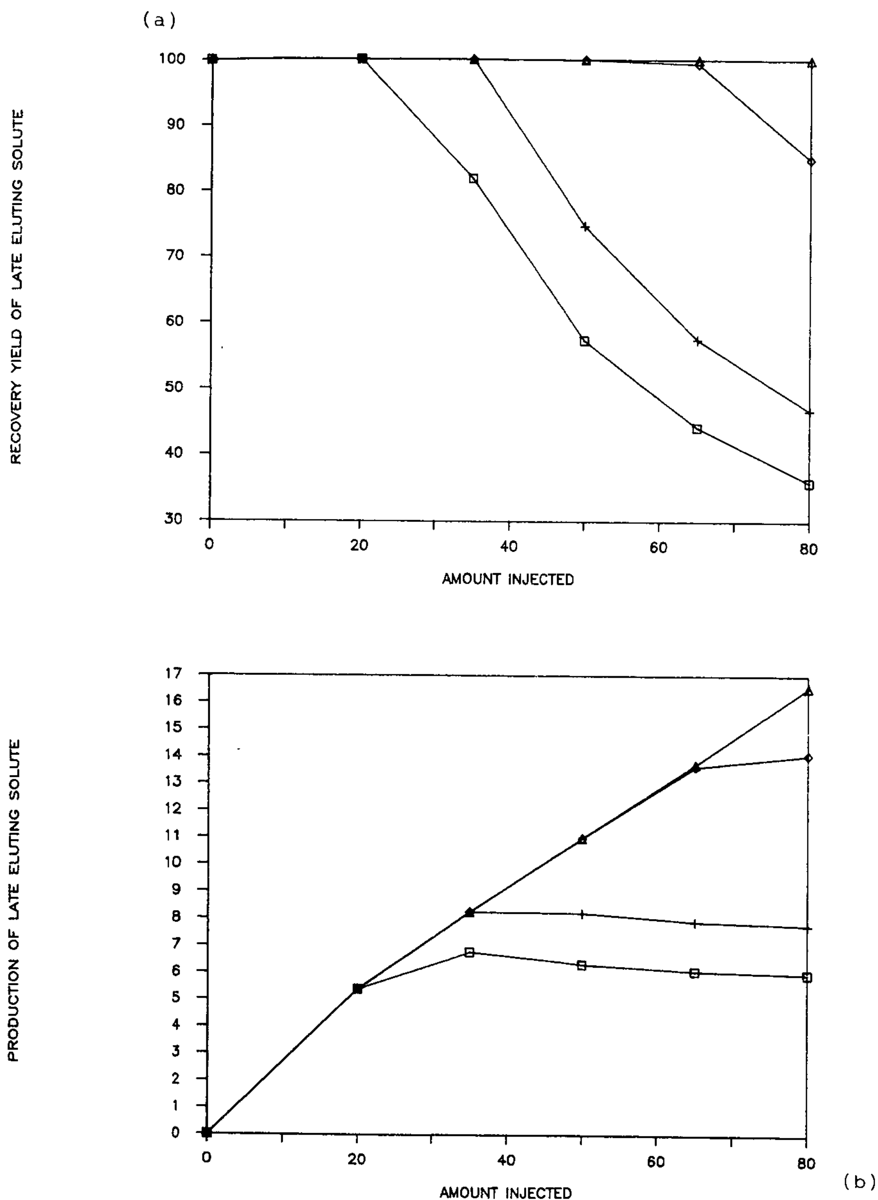

Fig. 8. As Fig. 7 for component 2. Key as in Fig. 4.

The only differences come from the relative intensities of these efffects. In order to achieve significant band overlap during most of the migration of the sample compounds along the column, the sample size must be increased rapidly with increasing α. Then, for a certain degree of band interference, the displacement and the tag-along effects become stronger.

This is observed when the separation layer between bands 1 and 2 for 1:3, 1:1 and 3:1 mixtures especially becomes narrower, and the tail of compound 1 behind

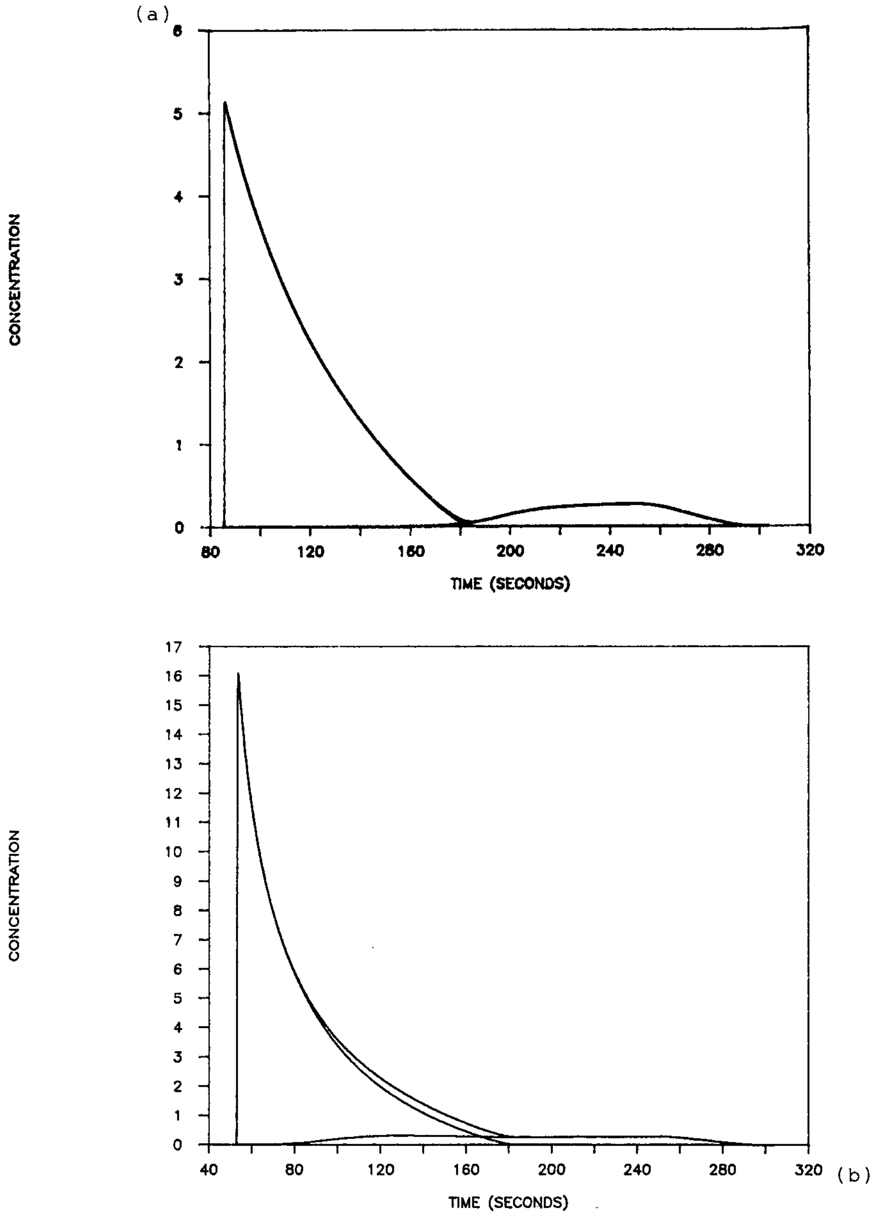

Fig. 9. As Fig. 6 for a 9:1 mixture.

this layer becomes smaller. This is also shown by the considerable tag-along effect resulting in the spread of the band of an impurity over a considerable range of retention volumes (see Fig. 9b, where the band of compound 2 goes almost from $k' = 1$ to 6). This is also illustrated by the important distortion exhibited by bands that are just resolved (resolution ≈ 0.9–1.2) at the column exit. Depending on the

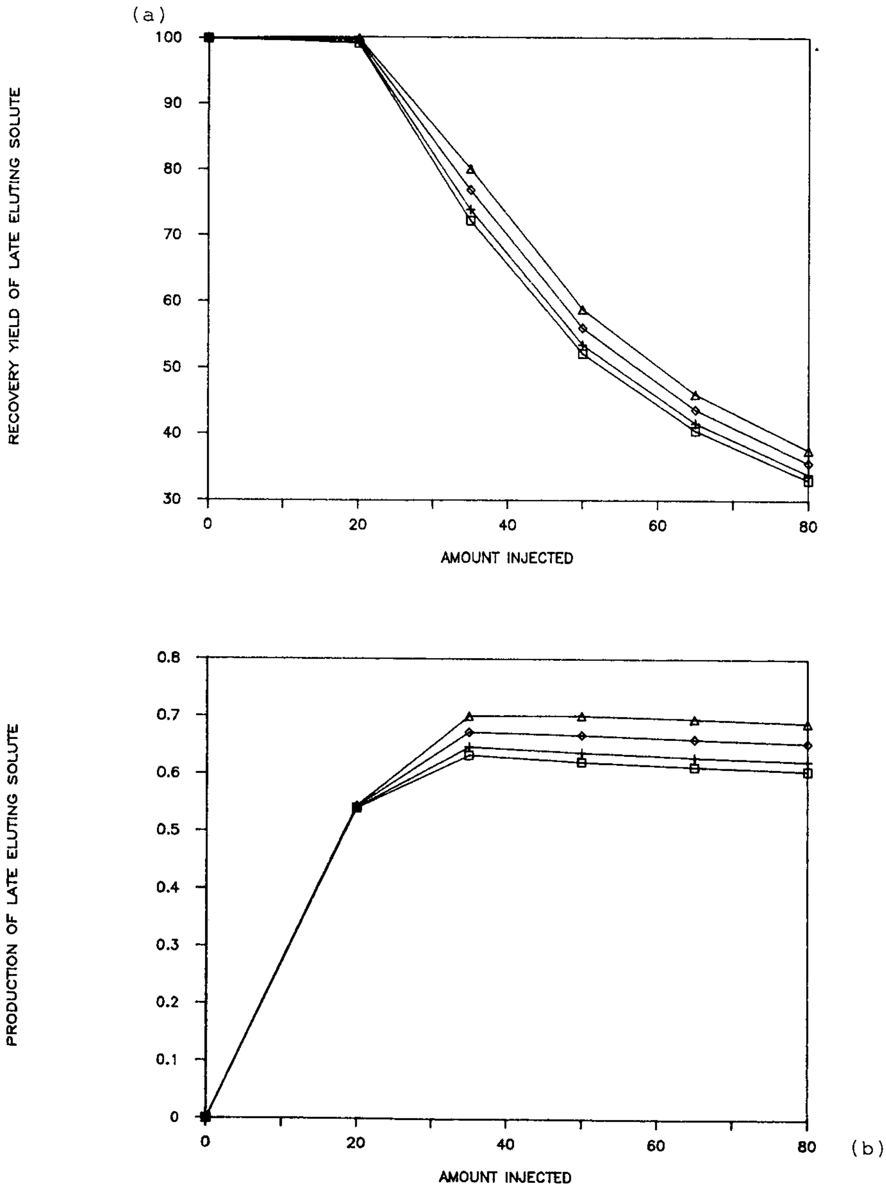

Fig. 10. As Fig. 8 for a 9:1 mixture. Key as in Fig. 4.

relative concentration, the effect is seen mainly either for the first compound (Fig. 1a) or the second compound (Fig. 9a).

The importance of the relative retention with respect to the optimal production rate of a preparative separation is considerable, at least when the two binary isotherms slowly diverge, as discussed here. This is illustrated by Fig. 11. Both the recovery and production rate increase rapidly with increasing α for both compounds

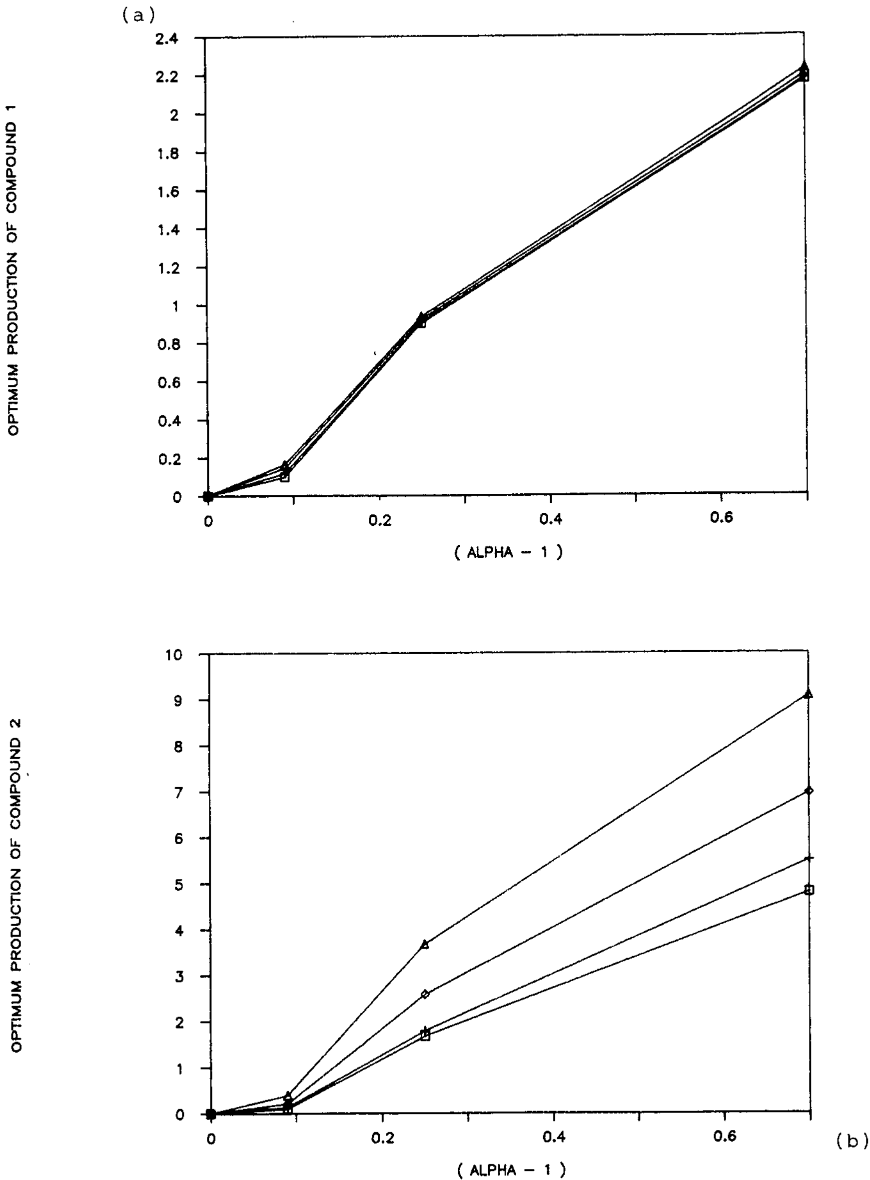

Fig. 11. Optimal production rate *versus* $\alpha - 1$ for a 1:3 mixture for (a) compound 1 and (b) compound 2. Key as in Fig. 4.

1 and 2. This result was obviously expected. It must be emphasized, however, that in all instances the production rate keeps increasing with increasing size whereas the yield decreases markedly. The maximal production is obtained for values of the yield that depend to some extent on the experimental conditions, but mainly on the relative retention (*i.e.*, *ca.* 90% for $\alpha = 1.7$ and *ca.* 70% for $\alpha = 1.25$).

The selection of the experimental conditions should stress the importance of selecting a chromatographic system in which the most important compound is eluted first (unless the equilibrium isotherms are concave), the binary (single component) equilibrium isotherms diverge and the relative retention at zero sample size is as large as possible. Then, the selection of the sample size will depend on whether a maximal production rate is sought or whether a compromise between the recovery and production rate is desired.

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COLUMN HARDWARE IN PREPARATIVE LIQUID CHROMATOGRAPHY WITH AXIAL FLOW

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SUMMARY

Scale-up of liquid chromatography (LC) for preparative work poses a number of problems. Problems concerning the column as the carrier of the stationary phase are discussed. Only axial column flow and LC with elution are considered (not radial flow, thick-layer, centrifugal techniques, etc.). The choice, design and technology of columns have mechanical aspects, but they are also dictated by the choice of stationary phase, by sample size requirements and by other chromatographic requirements. A distinction is made between laboratory-size and production-size instrumentation. The design, packing and use of laboratory-size preparative LC columns is not so very different from usual analytical LC practice. Larger-size instrumentation requires different approaches. A bewildering variety of larger-sized column designs is already commercially available. Dry-packing, slurry-packing, axial and radial packing or a combination of the two, chromatography with or without compression all are advocated. About 50 manufacturers of preparative LC columns were asked to provide their latest documentation on larger-scale systems. An analysis and survey of current commercial production-scale LC columns, based on the replies received, is presented.

INTRODUCTION

The increased interest in preparative liquid chromatography has recently been manifested by several events. Dedicated symposia have been organized on both sides of the Atlantic[1,2], a specialized journal has been launched[3], several books have appeared[4–6] and the numbers of papers and reviews on the subject has risen sharply. The present contribution to preparative LC concentrates on some physical aspects of the column as a carrier of the stationary phase. Stationary phases will not be discussed, or only to the extent that they influence the column shape. Radial-flow chromatography, thick-layer and centrifugal techniques, etc., will also not be considered.

Scale-up of LC for preparative purposes involves a number of choices concerning the column shape. The size, design and technology of columns have mechanical aspects, but they are also dictated by the choice of the stationary phase, by sample size requirements and, of course, by general chromatographic requirements. In particular, the column entrance and exit design must avoid peak broadening by unequal flow patterns.

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A clear distinction should be made between research and development preparative LC and process and production preparative LC. The first belongs in the laboratory, and the amount of compounds to be separated will generally be in the range μg to g. Often, the separated compounds are used for structural investigations by spectrometric techniques. The economics and engineering aspects of this kind of preparative LC are of secondary importance and any instrumentation which can accomplish the desired result is acceptable. Such preparative LC has been around for a long time. It has evolved very dramatically in the last few years. The second variety, or process and production preparative LC for commercial purposes, is a new development. This may cover much larger sample sizes (in the range kg to ton), and the economics are all-important. The difference between these two forms of preparative LC is reflected in the column design.

COLUMN DESIGN AND SAMPLE SIZE

For a long time, organic chemists have performed preparative chromatographic separations in glass tubes with gravity percolation of the solvents. The rule-of-thumb was to use 30 g of silica gel per gram of the mixture to be chromatographed[4]. The size of the column was adapted to the available sample size. With the advent of modern LC and the higher cost of the metal columns and packing materials currently used, the sample size today is adapted to the size of the available columns and instrumentation.

Separation is the goal. This is determined by the resolution, R_s

$$R_s = \frac{1}{4}\sqrt{N} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'}{k' + 1}$$

where N is the plate number of the column, α is the separation factor or the ratio of the capacity factors of the pair to be separated and k' is the capacity factor of the compound with the longest retention. While N is strongly influenced by the sample size, α and k' are practically not influenced at all. For a given separation problem the sample size is therefore mainly dictated by the efficiency/sample size dependence of the column. It should be stressed that this is for given conditions (fixed α) since, of course, other conditions (other α) may give very different sample size possibilities.

The usual ranges for the parameters of the resolution equation in analytical LC are: for N, 5000–25 000; for α, 1.01–2.0; and for k', 1–10. In this range, an increase of only 10% in α dramatically increases the resolution, while a 10% increase in the other factors has little or no influence. Since the value of α thus has the greatest impact on the resolution, it is essential to optimize α. If α can be increased above 2 or even higher, the separation becomes easy and sample sizes can be very large. This is not necessarily a desirable situation in analytical LC, because very large α may mean longer analysis times for the same column length. In analytical LC there is no need to aim for α values as large as 10–20, but in preparative LC these are very desirable. In preparative LC the α value is very important and should be as high as possible.

For a given chromatographic system, α, and also k' are in principle independent on the sample size. The plate number is the only other important parameter. Curves relating efficiency to the sample size have been discussed repeatedly in the literature. About twenty papers are cited in ref. 1. A knowledge of this relationship is important

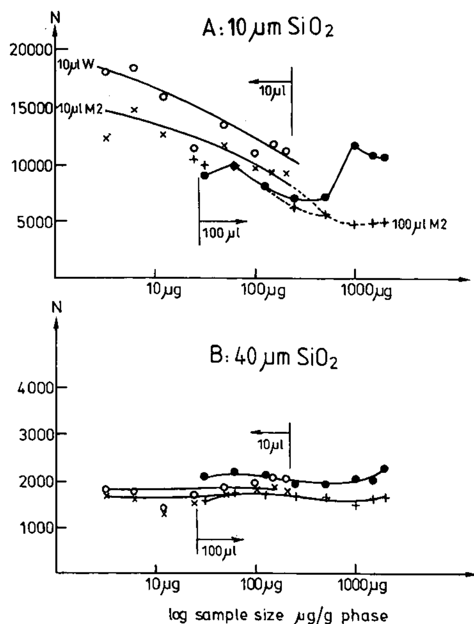


Fig. 1. Plate number per metre against log of sample size per gram of silica gel for normal-phase LC of 4-nitrophenol with 15% propanol in hexane. Sample injected in 10 or 100 µl. Circles: plate number calculated from the peak width. Crosses: plate number calculated from the second moment. Packings: (A) 10-µm RSil; (B) 40-µm RSil (RSL-Alltech Europe, Eke, Belgium). ○ = W (10 µl); ● = W (100 µl); × = M2 (10 µl); + = M2 (100 µl).

for the optimization of large-scale preparative LC. However, establishing such curves experimentally presents a number of problems. There is, *e.g.*, no consensus on whether to express the relationship on the basis of plate number or plate height. Some authors use a logarithmic scale for the sample size (sometimes expressed per gram of stationary phase) and/or for the plate number. How to measure the plate number or plate height is also a matter of controversy. From the purely practical standpoint, solubility and detection difficulties often make it impossible to cover the desired range of sample sizes.

Fig. 1 illustrates some of these difficulties. The difference between measuring the plate number by peak width or by the second-moment method is evident from Fig. 1A. This is not always so. In the present case, the non-ideal curve shape is attributed to anomalies in the adsorption isotherm of 4-nitrophenol. With small particles (10 µm), a wide range of efficiencies is available by simply changing the sample size. For larger particles (40 µm) the situation (Fig. 1B) is much less critical, but the efficiency is always poor. For µg sample per gram stationary phase (Fig. 1A), analytical efficiency may be achieved. With up to about 1 mg sample per gram stationary phase, acceptable chromatography is possible, with low-efficiency broadened peaks, but still with chromatograms resembling those in analytical LC. Whether the desired separation will still be possible at this sample load depends entirely on the problem (α value). However, in practice, much higher loads are often used. Organic chemists will not

hesitate to apply 10–100 mg of sample per gram of silica gel stationary phase. The peaks are so completely distorted then that they no longer appear as such on the chromatogram, which often can no longer be used as a guide in the separation. Displacement effects become important in this concentration range. Even larger loads are used in industrial applications. This then approaches selective filtration, rather than chromatography, but the hardware and technical approach are those of chromatography. Displacement chromatography can be aimed for deliberately, especially for dedicated production-scale applications. The column technology remains the same.

Resolution parameters are ultimately determined by the nature of the stationary and mobile phases. The sample size that can be separated in one experiment depends on the column volume. In modern laboratory preparative LC, with pressurized metal columns, most columns have an internal diameter (I.D.) of 7–10 mm and length 30–50 cm. Such columns will accommodate *ca.* 10–20 g of silica gel and can handle sample loads between 1 and 1000 mg, depending on the α value. A larger, much-used laboratory column is 25 cm $\times$ 2.2 cm I.D. and holds *ca.* 65 g silica gel or silica gel-based packing material. This size has to do with the fact that it is about the limit that can be: (i) handled by analytical LC instrumentation (flow-rate 10 ml/min is the optimum for such a column), (ii) packed efficiently with small particles by conventional methods, (iii) operated for a longer time without continuous column compression, and (iv) constructed with conventional ferrules and fittings.

In industrial or production preparative LC much larger columns are used. Sizes reported in the commercial literature range from 5 to 200 cm I.D. with variable bed lengths. These systems will accommodate, *e.g.*, 1–500 kg packing material, and the sample sizes that can be handled are in proportion. However, production preparative LC must not necessarily be linked to very big columns. Some compounds are used only in very small amounts, even in commercial applications, *e.g.*, biological preparations.

PACKING MATERIALS AND COLUMN DESIGN

Packing materials influence the column design not only through the α value which they determine, but also through their particle sizes. The particle size determines the packing procedure and the back pressure of the column. This again influences the physical aspect of the columns. With particles in the range 50–200 or even 200–500 μm, which are still used in some cases, packing can be achieved in the dry state by simply pouring the powdered packing material into the column. The back pressure of a column packed with such stationary phases is very small, and this considerably simplifies the requirements of column hardware. Any column tube material, even glass or plastic, can be used. With the recent tendency towards smaller particles, this has changed dramatically. The most popular particle size of packing materials is now in the range 15–20 μm. Currently, this seems to be the best compromise between cost, efficiency and instrument capabilities. Such particles cannot be packed in the dry state and require high-pressure slurry packing. Pressures of at least 10–50 bar are needed. For10-μm materials, which provide still greater efficiency, the packing pressure must be even higher. For analytical-sized columns (0.46 cm I.D.), the packing pressure with a 10-μm material is usually 400–600 bar. Naturally, the same pressure should also be applied to columns of larger I.D. if 10-μm particle-size packing materials are used. This

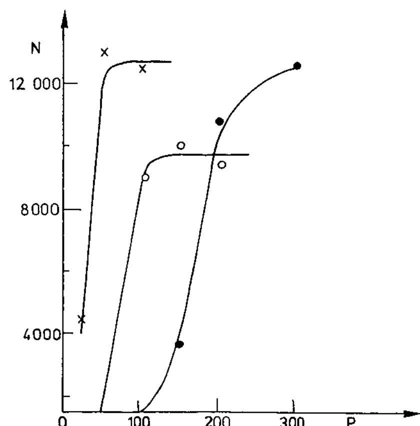


Fig. 2. Plate number for a 25 cm × 22 mm I.D. column packed with 10-μm reversed-phase silica gel (10-μm RSil-C18-HL-D) as a function of packing pressure, *P*, and slurry solvent viscosity, η. Solvents: ● = methanol (η = 0.60 cP); ○ = diethyl ether (η = 0.23 cP); × = pentane (η = 0.23 cP).

is not so easily accomplished. A stainless-steel cylinder of 20 cm I.D. with a wall thickness of 1 cm can withstand 100 bar, but not much more. With low-viscosity solvents, which increase the packing speed and the impact speed of the particles on the bed, slightly lower pressure may be adequate[7]. This is shown in Fig. 2 for 2.2 cm I.D. columns. While a 10-μm reversed-phase derivatized silica gel would be packed in analytical-sized columns, at 400–600 bar in, *e.g.*, a methanol or carbon tetrachloride slurry, a pressure of 50 bar is sufficient for low-viscosity pentane as the slurry solvent. A word of warning is necessary. Slurry packing of columns of larger I.D. is still an arcane art. The results presented in Fig. 2 may not be obtainable with another/different packing material.

A fused-silica capillary with an I.D. of 320 μm as used in micro LC[8] can withstand up to 500–800 bar of slurry-packing pressure. At 1000 bar, the fused-silica

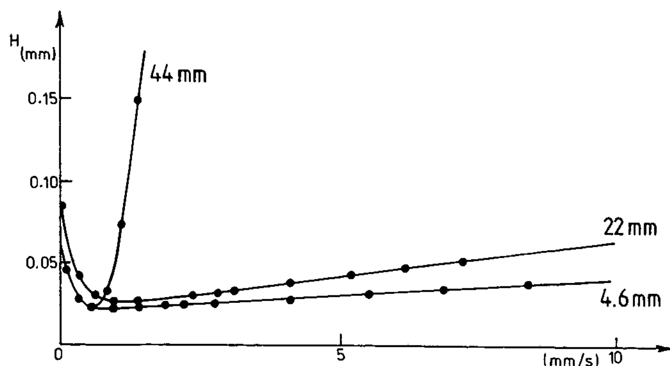


Fig. 3. Influence of column I.D. on the *H*/*u* curve (*u* in mm/s) for small packing particles. Stainless-steel columns of 25 cm × 4.6, 22 and 44 mm I.D. Stationary phase: reversed-phase silica gel (10-μm RSil-C18-HL-D). Mobile phase: water–acetonitrile (25:75) with pyrene (k' = 6) as sample.

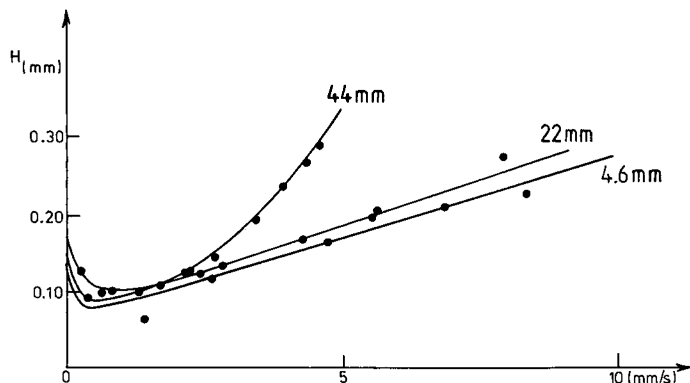


Fig. 4. As in Fig. 3, but with coarser particles (20-μm RSil-C18-HL-D).

column may explode. Stainless-steel analytical LC columns with an I.D. of 0.46 cm are frequently packed at 600–800 bar. They have a wall thickness of about 1 mm. With columns of much larger I.D., in the range 20–50 cm, the wall thickness has to be much larger, if not for packing, then for just operating the columns, which may also require rather large pressures. While the wall strength is not so important for analytical LC, this is not true of preparative LC. Large-scale preparative LC columns must be very heavily walled if they are to be used with small-sized particles. The particle size of the packing material determines the working pressures of the columns and this, in turn, influences the mechanical aspects of the columns. Safety considerations are most important. Rupturing an analytical LC column may not be very dangerous, but this is certainly not true for larger systems. With the modern tendency towards smaller-particle packings and therefore higher pressures, this point should not be ignored.

The particle size of the packing material is also important for the frictional heat generated in the column when the solvent is forced through it. The frictional heat depends not only on the particle size but also on the viscosity of the solvents. When viscous mixtures of methanol–water or acetonitrile–water are used in reversed-phase systems, the frictional heat is more important than in normal-phase LC systems where

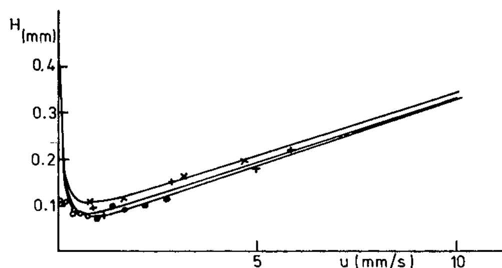


Fig. 5. *H*/*u* curves for columns of various I.D., packed with normal-phase silica gel (Prep-RSil of average particle size *ca.* 20 μm). The reduced plate height is about 3–4 for all systems. No deviations are observed, also not for the "slurry"-packed 4.1 cm I.D. column. Floating-piston-packed and compressed columns do slightly better. The I.D. of 4.1 cm is still modest. Columns: × = 0.46 cm I.D.; + = 4.1 cm I.D., slurry packed; ● = 4.1 cm I.D., floating piston; ○ = 10 cm I.D., floating piston.

low-viscosity solvents are used. The frictional heat would also be more important in columns of large I.D. where heat dissipation is more difficult. These remarks are illustrated experimentally by the curves of plate height (H) versus linear velocity (u) of Figs. 3–5.

Guiochon and Colin[9] state that the shape of the H/u curve is not affected by the I.D. in the range of 1–10 mm. This can be extended to 2.2 cm I.D. columns, even for 10-μm particle and reversed-phase systems. However, in columns of larger I.D. deviations from the H/u curve can occur, as shown in Fig. 3. With 20-μm particles, the deviations are less pronounced, as shown in Fig. 4, but they are still noticeable. The H/u curves in Figs. 3 and 4 can be reproduced repeatedly on the same column, but for the columns of larger I.D. this is true only for a few cycles. The deviations are ascribed to frictional heat, which acts in two ways. First, the flow profile is deformed immediately, because the solvent viscosity is not the same throughout the whole column cross-section. Secondly, the quality of the packing is destroyed, not immediately, but still fairly rapidly. The stainless-steel mantle and the packed bed do not have the same thermal expansion, and this leads to voids and gaps in the packing, specifically at the column wall. With columns of still larger I.D., *e.g.*, 20 cm, this defect can even be visually demonstrated. A freshly packed column has no visible gap between the packed bed and the wall, while such a gap may appear in a column which has been used under conditions where frictional heat effects can be expected. In normal-phase systems, the frictional heat should be less important, because of the lower viscosity of the solvents, resulting in a lower working pressure. This is shown in Fig. 5, where H/u curves for such chromatographic conditions are seen to be without deviation for a 4.1 cm I.D. column. With a 10 cm I.D. column, abnormal H/u curves are obtained, even in normal-phase LC. Columns of larger I.D. are thus unstable, if they are packed with 20-μm and finer material. The line between normal and abnormal H/u curves is not clear-cut. This is obvious, considering the above remarks, and explains controversial opinions about column stability in the field of preparative LC. It is possible to pack even 20 cm I.D. columns of conventional design efficiently with smaller particles, but the columns are not stable. We have repeatedly packed such columns. Even under favourable conditions, *e.g.*, with 20-μm silica gel and hexane as the solvent at a moderate flow-rate, the system, which originally had a good reduced plate height, could hardly withstand more than a few hours of use.

Thus, it is a fact that columns of large I.D. of the conventional type are less stable than analytical-sized columns. Frictional heat is one of the reasons. Through repeated thermal expansion and contraction the packed bed changes, and a void is produced at the top and along the walls of the column. If the packing pressure applied to such systems is too low, this also leads to instability. The pressure surges of the pump during chromatography are probably not compensated by pulse dampeners (as in analytical LC) and this too has a deleterious effect. At very low flow-rates, as used with soft packing materials, or with coarse packing materials, these problems are less important, but in modern high-speed small-particle LC they are. The way to solve this instability problem is to adapt the column volume continuously to the changing packed-bed volume. This is possible with judiciously applied continuous column compression during chromatography. An alternative is to compress the column bed intermittently between consecutive chromatographic experiments. The pumping of liquid through the column during chromatography also compresses it, but this would tend to

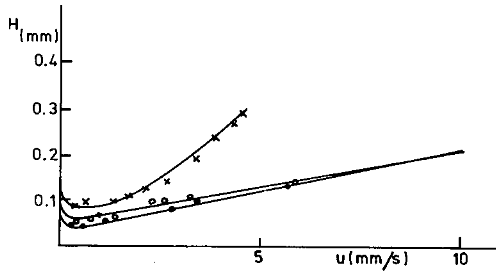


Fig. 6. As in Fig. 5, but with reversed-phase silica gel (Preparative RSil-C18-HL-D). With the higher-viscosity solvents, deviation occurs in 4.1 cm I.D. columns which are slurry-packed. The floating-piston-packed and compressed 4.1 cm I.D. column behaves normally. Columns: ● = 0.46 cm I.D.; × = 4.1 cm I.D., slurry packed; ○ = 4.1 cm I.D., floating piston.

destabilize the system, as mentioned, because it does not change the column volume. The compression should reduce the column volume or adapt the available column volume to the changing volume of the packing material. That this is effective is shown in Figs. 5 and 6 for 4.1-cm columns, even when packed with reversed-phase 20-μm material. While these conditions lead to abnormal H/u curves without compression (see Figs. 4–6) they do not when the column is compressed. Column compression is therefore essential for large-scale LC with relatively small-sized particles.

The reduced plate heights in Fig. 5 are about 3 for all systems. The results are slightly better with the floating-piston compression system. For the columns of larger I.D., the very high flow-rates, which are desirable for establishing the H/u curve over its entire range, could not be achieved with the available pumps. With the reversed-phase columns in Fig. 6 the reduced plate heights attainable were even slightly better and close to 2, although the packing material showed quite a spread in particle size.

Whether a column will be compressible or not affects its design considerably. Since column compression can be avoided by using coarser particles, normal-phase

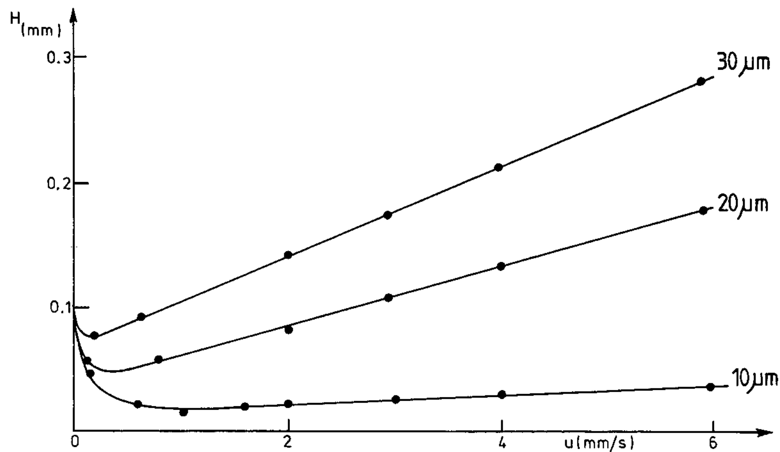


Fig. 7. H/u curves and particle size. 25 cm × 4.6 mm columns, packed with 10, 20 and 30 μm RSil-C18-HL-D reversed-phase silica gel.

solvents and slow eluent speeds, why go to the trouble of working with much costlier compressed systems? Fig. 7 shows why relative smaller particles should be chosen, if possible, as a function of cost and instrument capabilities. Such curves are well known in both gas chromatography (GC) and LC. The steepness of the curve with increasing flow-rates increases dramatically with larger particles. This is even more pronounced for still larger sizes than those shown in Fig. 7. At the optimum flow-rate the 30-μm material is only three times less efficient than the 10-μm column, and this would seem to be acceptable. However, the flow-rate at the optimum for the 30 μm material is very low. At the more usual and practical flow velocities of 4–6 mm/s, the 30-μm material is much less efficient than the smaller-sized packings. Differences up to a factor of 10 are then observed. Since smaller particles are preferable, column bed compression in larger systems is thus of real importance. Compressed columns have been commercially available for a long time. The above arguments and discussion could therefore be deemed to be unnecessary. However, the recent literature shows that the importance of compression in preparative LC is still not generally recognized. Compression is considered to be necessary for packing the column, which is of course correct, but it is also necessary during chromatography or intermittently to adapt the column bed volume. Whether continuous compression is needed or intermittent compression sufficient is not known. This may well depend on the particular conditions. If really high efficiency is needed, smaller particles, longer columns and higher pressures will be required. Under these conditions, for packing and during chromatography, we believe compression is necessary.

COMPRESSION AND COLUMN DESIGN

For columns up to about 2.2 cm in I.D., the approach to column design, packing and handling can be the same as for the conventional columns, as already mentioned.

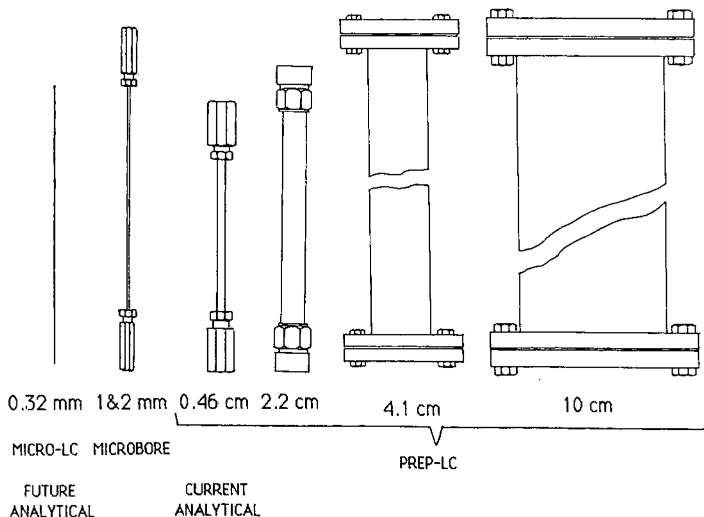


Fig. 8. Schematic drawing of columns of various I.D. (without compression). Up to 22 mm I.D. a nut-and-ferrule system can be used. Columns of larger I.D. must be flanged. Current analytical columns are the small preparative (Prep) LC columns of the future.

However, the ferrules, needed to keep the fittings in position, must be very carefully adapted or else they will slide off the column under the pumping pressure. This difficulty increases rapidly with increasing column diameter and 2.2 cm I.D. is about the limit. A drawing of such a column is shown in Fig. 8. Up to that I.D., column bed compression is not needed and reasonably stable columns can be made without compression. Still, such columns should be handled carefully and not be subjected to very high pressures. Above that I.D., the columns must be terminated and closed in a different manner (with flanges, as also shown in Fig. 8). They also require bed compression, at least for packing, if not during the chromatography to extend the column life expectancy.

Compression can be accomplished either axially or radially. Instruments based on both approaches have been patented and are commercially available from a number of manufacturers. Axial compression can be exerted from above with an adjustable column head (Axxial, Cedi, Merck, Rainin) or from the bottom with a fixed or floating piston (Axxial, Prochrom). A floating piston requires less head space and seems particularly attractive. Radial compression can be achieved with a gas or liquid between a flexible mantle and the column or with prongs (Waters). A combination of axial and radial compression is obtained by inserting a wedge or plunger in the centre of the column top, which is screwed down as the column conditions require (Separations Technology, SepTech). Another axial compression system involves prongs and autocompression from above (Cedi). In the Cedi approach, the plastic packing material cartridges are so heavily walled that the radial compression is thought only to hold these columns and not to compress them. These compression concepts are discussed further and illustrated in Figs. 9–19 with documentation obtained from the manufacturers. Some systems combine compression packing and chromatography under compression (Axxial, Cedi, Prochrom). Others use compression only for packing (Amicon, HT-Chemicals). Still others offer both possibilities (Merck, Varex, Waters). The Rainin and Separations Technology techniques achieve compression in quite a different way. Time will tell which technique is preferable. With really coarse particles no compression is needed, and very simple, not so sturdy instrumentation will be adequate. For intermediate situations, compression for packing only may be sufficient. It is also important how easily and rapidly a used column can be re-compressed. For small-particle, efficient, production-scale preparative LC with long column life expectancy, compression is needed, we believe, not only for packing, but also during chromatography.

PACKING COLUMNS OF LARGER I.D.

The packing technique for columns of larger I.D. depends on the particle size and the column hardware. Very large particles (30–50 μm and above) can be dry packed (the packing material being evenly poured into the column). For very wide 1–2 m I.D. columns, the packing material may be added through a sieve. Smaller-sized particles are either slurry-packed in the conventional way, as for analytical-sized columns, or they are compression-packed. Pumping a slurry at the required speed into a really large column (packing the column like an analytical-sized one) is very difficult. Few pumps have the required capacity. Special know-how must compensate for this (see Fig. 2). Slurry packing is, in fact, forced filtration which eventually results in

a packed bed. Compression packing physically squeezes the packing together. Compression packing a column is rapid and easy, especially with a cartridge system. It is always amazing to see how quickly the column packing is actually achieved with a piston compression instrument. Some manufacturers compression-pack the columns in a separate instrument (a packer) and then take the column over to a chromatographic unit which does not apply extra compression during chromatography (Amicon, HT-Chemicals). Other systems use compression for packing and continuously during chromatography. With other systems, compression is actuated intermittently as the column conditions require. Commercial publicity stresses that stationary phases can be reused many times in preparative LC. In this respect, the brittleness of the particles is important. All packing techniques, and certainly those in which physical compression is used, will crush particles. This may create so much "fines" that permeability problems arise. The hardness of the particles is therefore an important quality criterion. This hardness is indeed very different for different silica gels. Better means for determining the hardness of packing particles ought to be developed. Partly crushed materials can, in principle, be reclassified. For silica gel this can be accomplished without problems. For derivatized silica gel, the breaking of particles produces new surfaces with new silanol functions that are not derivatized. This changes the chromatographic characteristics and has to be avoided.

LARGER COMMERCIAL SYSTEMS

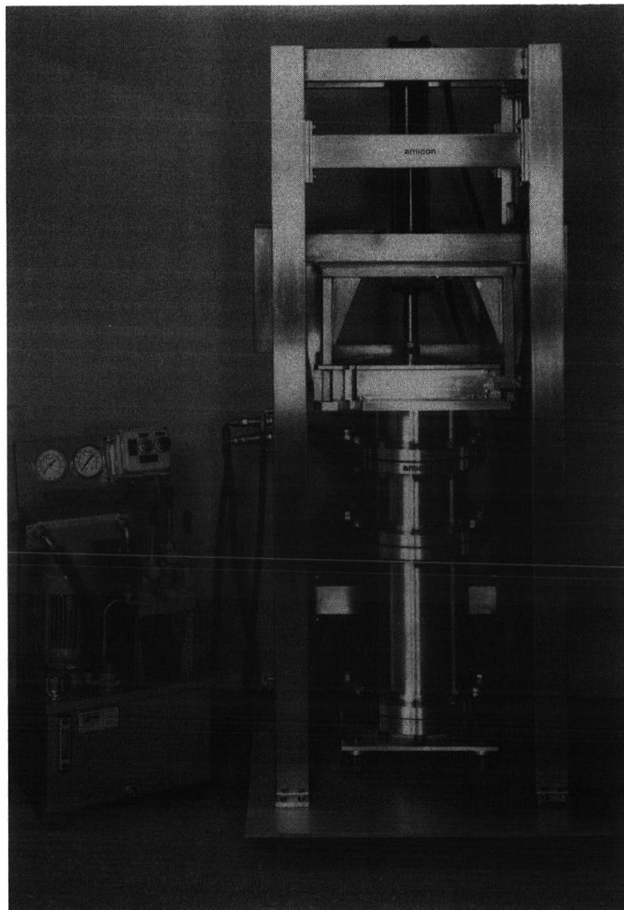
At the end of 1987 about fifty manufacturers of preparative LC columns were asked to communicate their latest developments in the field. Not all of them have replied nor are all specifically active in preparative LC column production. The following list is based on the replies received. Only systems with column I.D.s larger than 5 cm are mentioned. Many other manufacturers produce smaller columns for laboratory preparative LC.

Amicon (Upper Mill, Stonehouse, Gloucester, GL10 2BJ, U.K.)

Amicon offers a wide range of preparative LC columns: in glass up to 4.4 cm I.D., in high-pressure (135 bar)-resistant stainless steel up to 30 cm I.D. and, for lower pressure, production preparative LC on the largest scale, columns with adjustable bed length of 200 cm I.D. Amicon separates the packing procedure from the actual chromatography. The K Prime 3000 columns are thus not "compressed", but the adjoining "packer" (N Pack 3000) does use ram compression. This should facilitate column regeneration. Fig. 9 is a picture of the Amicon N Pack 3000 packing station, showing the ram piston just above the column.

Axxial (44, Rue Maurice de Broglie, F-92800 Aulnay-Sous-Bois, France)

Axxial is a new firm continuing the activity of Jobin-Yvon and Chromatofield. Their Modulprep has been commercially available for a long time. This is both a packing and a chromatography station, which has now been joined by a newer Pilotprep for columns up to 10.9 cm in I.D. An interesting feature is the compression of the column bed from both sides with movable pistons. The Pilotprep separates the packing and chromatography functions. The principle is illustrated in Fig. 10. The column is also axially compressed during chromatography. As a result of the long



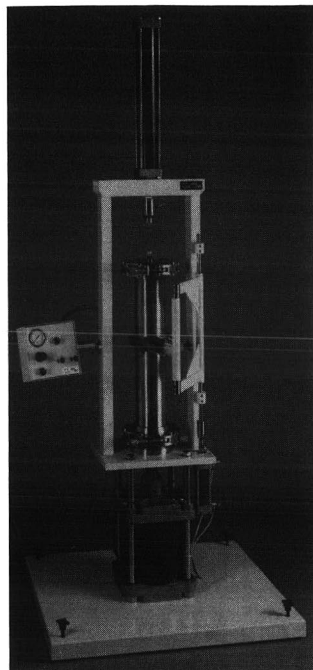


Fig. 9. The N Pack 3000 packing station of Amicon. This device is used for compression packing and for regeneration of columns.

Fig.10. The Axxial Pilotprep system, with which packing and chromatography are not carried out on the same column-holding hardware.

experience in the preparative LC field of Jobin-Yvon, a long list of applications is available.

Büchi (Meierseggstrasse, 40, CH-9230 Flawil, Switzerland)

Although the Büchi system involves glass columns, the approach is that of modern LC. The highest allowable pressure is given as 40 bar, but this seems high for glass and must not be without danger. So is one of the packing procedures described with pressurized gas. Büchi is, of course, aware of this and uses a plastic protective coating over the columns. The largest I.D. available is 10 cm. Normally, Büchi columns are not "compressed". However, a most recent version of the 7 cm I.D. type has a top piston for bed compaction. Glass has its advantages: its very smooth surface makes packing easier, and visibility and high chemical inertness are also desirable. In some cases, inertness may be a most important factor.

Cedi (Route des Usines, F-65300 Lannemezan, France)

This newcomer to the field uses top-applied axial (auto)compression on a piston, sliding in heavily walled plastic cartridges. The cartridges are held in a metal device, which closes with "prongs", as in a radial compression instrument. A specially designed inlet device diverts the eluent in such a way that it pressurizes the column-top piston, hence the name "autocompression". The system can be used with either one or two cartridges. The first cartridge can function as a guard column or can contain a different stationary phase to increase the separation specificity. The double-cartridge approach also allows recirculation without the need for the sample to go through the pump. Recirculation is a most attractive feature, deserving of more attention. Figs. 11 and 12 illustrate the Cedi technique. Columns up to 10 cm in I.D. are available.

Dorr-Oliver (77 Havemeyer Lane, Stamford, CT 06904, U.S.A.)

Dorr-Oliver supplies proprietary-design hardware for columns up to 1 m in I.D. A range of packing materials is available, and the packing technique and know-how for columns of large I.D. is transferred to customers. Compression is not mentioned. Dorr-Oliver states that each column I.D., and each particle size, requires a different packing method and that therefore optimum results can be achieved only in close cooperation between the customer and manufacturer: "We have chosen not to supply column packing systems to the market, because aspects of our technique ... require considerable know-how and experience". The smaller-sized columns are offered with a guaranteed plate number, *e.g.*, 22 000 for 1 m × 7.5 cm I.D. column packed with 10/20 μm octadecylated silica gel.

HT-Chemicals (4221 Forest Park Bvd, St. Louis, MO 63108, U.S.A.)

This firm has a range of stainless-steel columns up to 10.2 cm in I.D. Larger systems can be provided on request. A particularity of HT-Chemicals is their use of a SCRAM (slurry compression ram) for packing the columns. In actual LC, the columns are used without compression. A packing station is available. This can probably also be used for chromatography.

Merck (Frankfurterstrasse 250, D-6100 Darmstadt, F.R.G.)

The new, large Merck columns (Prepbar) are used with axial compression, applied at the top. This "compression" is continuous when the packed column is used in the Merck preparative chromatograph, but the packed columns can also be used in other instruments. In this case, the packing can be recompressed intermittently whenever necessary or at regular intervals, by tightening the bolts at the column top, thus adapting the column volume to the packed-bed volume. A range of Merck columns is shown in Fig. 13. The largest columns in this series have an I.D. of 10 and 20 cm. Merck advocates the use of cartridges and offers a wide range of packing materials. Cartridges somewhat restrict the free choice of the operator (this should in our opinion not be overestimated), since they determine the column length, but they have the advantage that the large amounts of silica gel can be cleanly handled. Fig. 13 also shows some large columns without a compression head device. In addition to these higher-pressure systems, Merck still advocates their Lobar non-compressed low-pressure glass columns. A recent review illustrates that, for laboratory applications, they can still be useful[10].

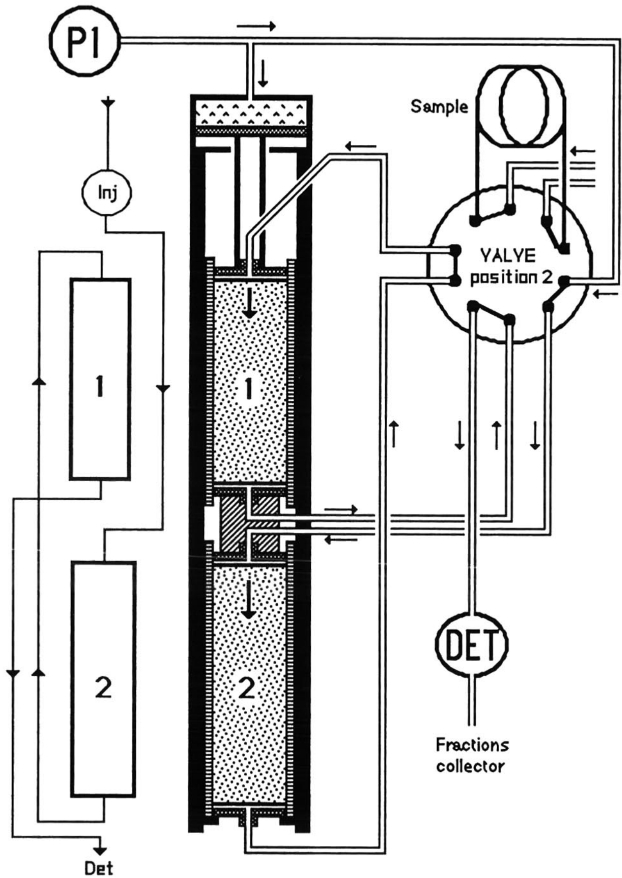


Fig. 11. The Cedi system. Two cartridges can be used either in series or in bypass. The Autocompression system is also shown. PI = Pressurized inlet; DET = detector; 1 and 2 = cartridges.

Pharmacia (Björkgatan 30, S-75182 Uppsala, Sweden)

The latest product from Pharmacia is the BioPilot concept. This preparative liquid chromatograph is quite different from the others in the sense that it involves glass and plastic columns, only works at a maximum pressure of 20 bar and strongly emphasizes biocompatibility. The size of the packing materials, like Sepharose and Mono Q beads, is relatively large and they are sterilized with strong sodium hydroxide solutions (something which is not possible or mentioned for other systems). Preparative LC is carried out in 6 cm I.D. columns and thus has the large capacity of production-scale preparative LC.

PPG Industries (P.O. Box 2844, Pittsburgh, PA 15230-2844, U.S.A.)

The latest in column technology comes from the Fiber Glass Research Center of PPG. They state their case as follows: "Column packing of aligned fibrous-cylindrical silica gel overcomes the limitations of particle-packed columns. These columns

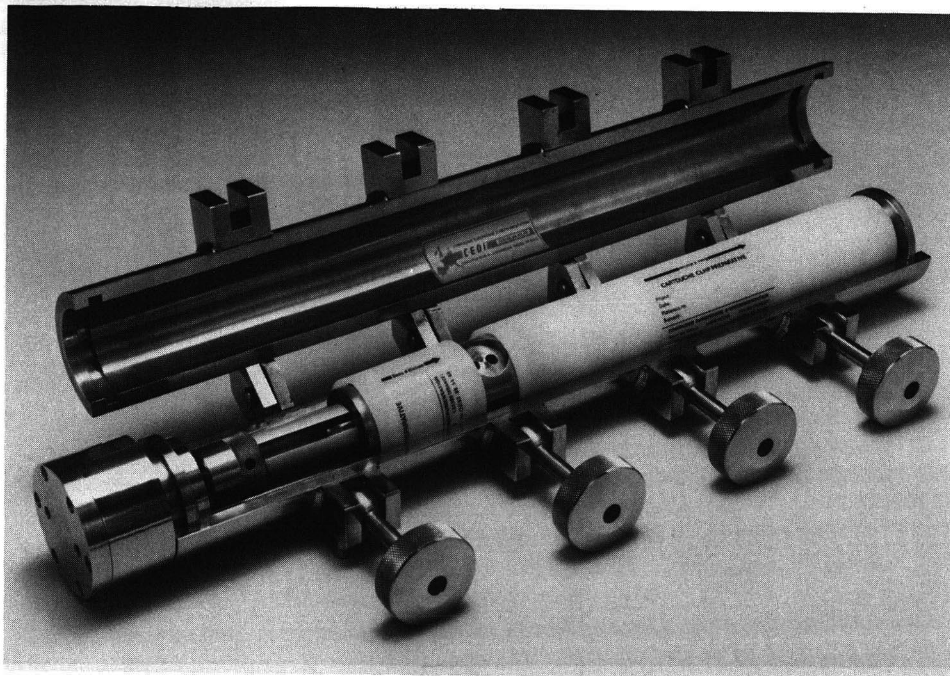


Fig.12. The metal holder of the plastic cartridges of the Cedi system.

combine high packing density with very good permeability. Techniques have been developed for reducing fiber variation and packing inconsistencies". PPG announces columns with I.D. of 7.5 cm. Such columns would not need "compression" techniques. It will be interesting to see how this approach develops.

Prochrom (Chemin des Blanches-Terres, BP 9, F-54250 Champigneulles, France)

Prochrom (formerly Chromatelf) offers 20, 30 and even 60 cm I.D. axially compressed columns with all necessary ancillary equipment. The advantages of this system are that the user packs his own columns and that various column lengths are thus available. Packing materials can be reused many times. The choice of packing material is also freer than with other systems involving cartridges or pre-packed columns. Prochrom obviously also has much experience with these largest I.D., commercially available, compressed production preparative LC columns. Fig. 14 is the by now well known illustration accompanying Prochrom advertisements. The same set-up is used for packing and for chromatography, which is thus carried out under continuous compression. Note that the Prochrom systems require hall space for their location. In these Prochrom columns of large I.D., the piston actually moves slightly up and down during chromatography. This is accompanied by a characteristic noise and is a clear indication that the packing is "alive" and that continuous bed volume adaptation or "compression" is needed.

Fig. 13. Merck columns, which are probably packed at very high pressure. The column top can be screwed in to actuate column compression.

Rainin (Woburn, MA 01801, U.S.A.)

The *dynam*ic *ax*ially compressed or Dynamax system of Rainin is based on axial compression by screwing down the whole column top (Figs. 15 and 16). The aim is to eliminate the voids that could develop at the column top. As the Rainin literature puts it: "As large volumes of mobile phase pass through, the cumulative effects of infinitesimal dissolution of packing material and minute pressure pulsations, no matter how small, eventually take their toll. The ideal bed structure disappears. Small

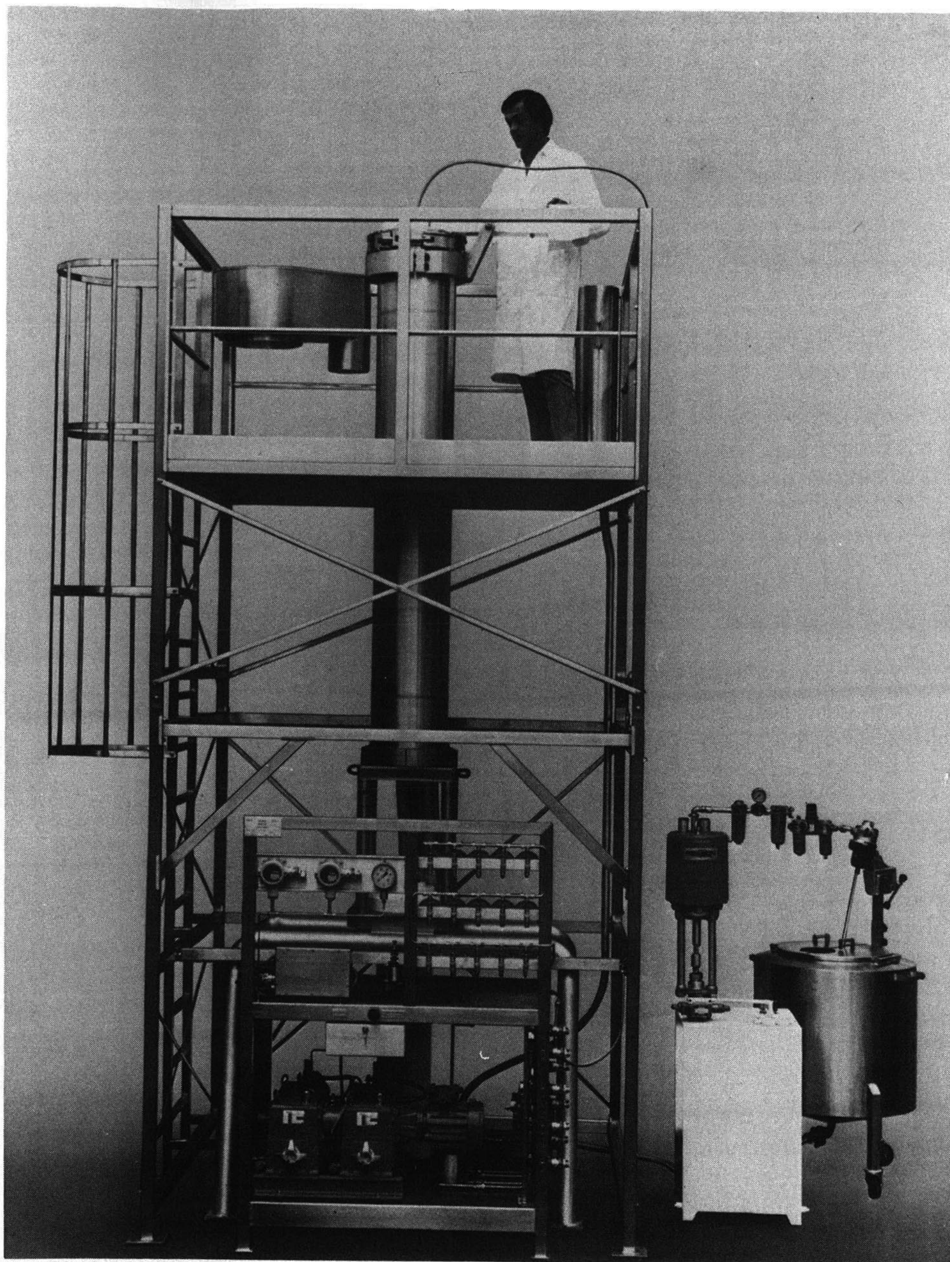


Fig. 14. The set-up for a 30 cm I.D. Prochrom axially compressed system. The required height for this instrument must be considered.

voids form at the head of the column. Efficiency and symmetry decline, and the column becomes unusable". Rainin applies its technique to analytical as well as to larger column sizes. The most recent Rainin preparative LC column has an I.D. of 10 cm with

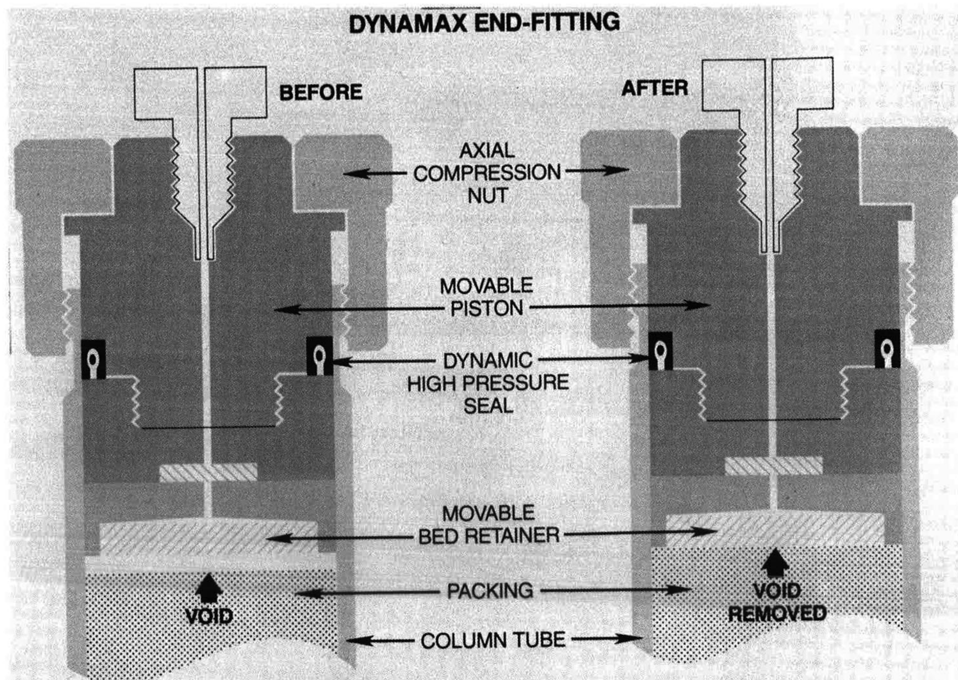


Fig. 15. The Rainin system. The whole column top is screwed down to reduce voids which may appear at the column top.

a bed length of 10 cm. This handles multigram sample sizes. Whether this is considered production scale or not depends on the problem, of course. The Rainin literature shows some examples of scaling-up experiments, for which the absolute identity of the analytical and preparative LC tracings is remarkable. Usually, changes in column dimensions introduce unavoidable chromatographic pattern changes.

Separations Technology (SepTech) (P.O. Box 63, 2 Columbia Street, Wakefield, RI 02879, U.S.A.)

This compression system inserts a wedge or plunger into the column. As a function of diminishing performance, this plunger is screwed down, as shown in Fig. 17. Compression is exerted at the same time in both axial and radial directions. Separations Technology introduced its Macrobore Annular Expansion methodology at the 1987 Pittsburgh Conference. Good chromatograms were shown at 200 ml/min flow-rate. This indicates a column *ca.* 10 cm in I.D. The largest column is claimed to hold 40 kg of packing material. With the apparent density of silica gel (about 0.5) this corresponds to a column of 70–100 cm × about 30–40 cm I.D. Documentation and/or a prototype of such a large SepTech Macrobore A/E instrument is not yet available.

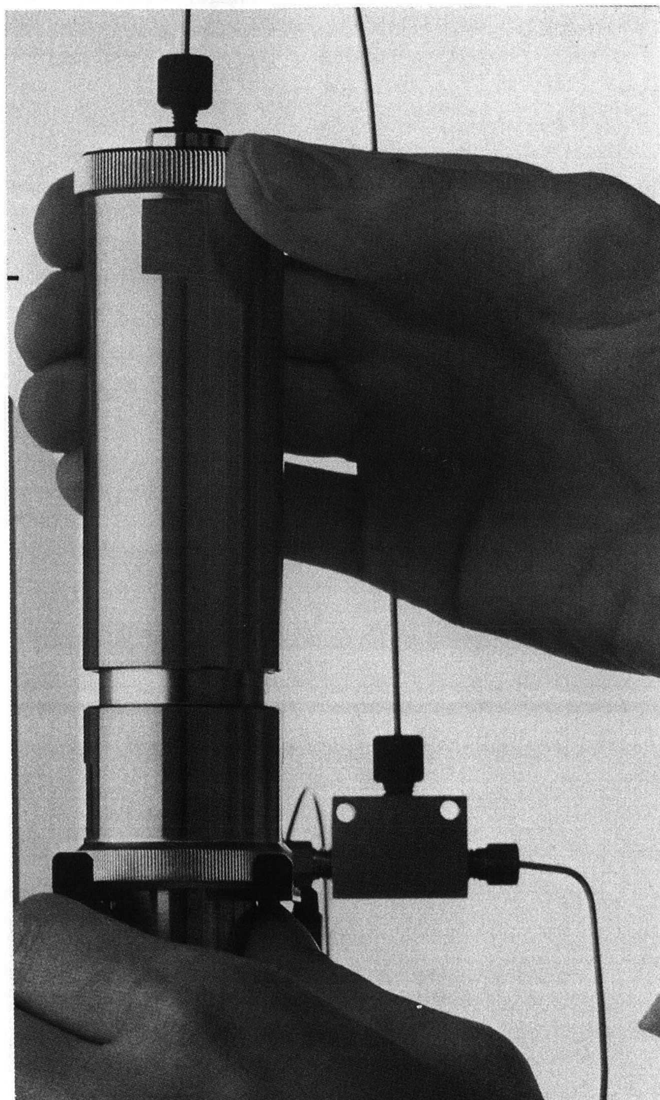


Fig. 16. Compression of the column top in the Rainin system can be achieved by hand tightening.

Varex (12221 Parklawn Drive, Rockville, MD 20852, U.S.A.)

The Varex Versa series covers a wide range of preparative LC columns, from the laboratory-range preparative LC 2.5 cm I.D. columns to production-LC systems with 30 cm I.D. columns. These are non-compressed systems. Varex offers a very wide range of pumps, detectors, injectors, collectors, gradient, recycling facilities, etc. High-performance preparative liquid chromatography at Varex has become HP^2LC which reminds us of the $(GC)^2$ of some years ago.

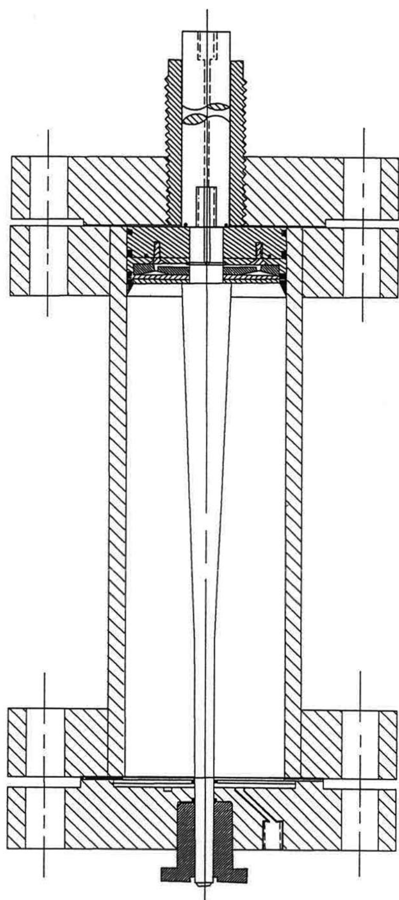


Fig. 17. Separations Technology (SepTech) system. The bottom screw can draw in the tapered plunger, thus achieving axial and radial compression.

Vydac. Division of the Separations Group (17434 Mojave Street, P.O. Box 867, Hesperia, CA 92345 U.S.A.)

Although this firm has no literature presently available on columns of larger I.D., it has started to offer 5 cm I.D. columns, pre-packed with its materials. Vydac specializes in the separation of polypeptides and other biomolecules. This particular field is bound to become more important with the development of biotechnologies. The success of recombinant DNA techniques will depend on good preparative LC. We feel that this particular point has so far not received the attention it deserves.

Waters (34 Maple Street, Milford, MA 01757, U.S.A.)

Waters, which is now a division of Millipore, is well known for its radial compression systems. Its earlier Prep LC/System 500 is probably the most popular preparative chromatographic system at this time. It is based on rather coarse packing materials, even in the 100-μm range. However, Waters has greatly diversified in the

Fig. 18. The Kiloprep Waters system, with 20 cm I.D. columns, which involves cartridges and axial compression.

preparative LC field and now offers even non-compressed metal columns, up to 5 cm in I.D. Glass columns, very small particles and a very wide range of packing materials are also available. Chromatographers have stated (lately, *e.g.*, at the discussion sessions of the Baden-Baden 1988 Prep-LC meeting) that the Waters columns perform as well with as without radial compression. This, we believe, is true only for very large particles, but then the original compressible Waters cartridges were filled with very large, about 70-μm, particles.

The successors of Waters 500 are called Delta Prep and Kiloprep. The largest Waters columns have an I.D. of 20 cm and involve cartridges and radial compression. Precise details on the column dimensions and technology for the larger systems are absent from the Waters literature. Fig. 18 shows the Kiloprep (or largest) system with 20 cm I.D. columns.

Whatman (9 Bridewell Place, Clifton, NJ 07014, U.S.A.)

Whatman offers a stainless-steel Magnum 70 column, 100 cm × 7 cm I.D. This column is intended for packing with Partisil 40. It is not a compressed column, although Whatman has a compression technology (WCS or Whatman Compression Screw). Whatman also offers a glass-reinforced, polymeric Prep-25 column for cellulosic media, with an I.D. of 45 cm and a bed 16 cm deep. This "pancake or waffle"-type column is something we will probably see much more of in the future (Fig.19). The logical endpoint of the quest for high efficiency and large capacity is indeed the use of small particles in a wide and short column. Another reason for

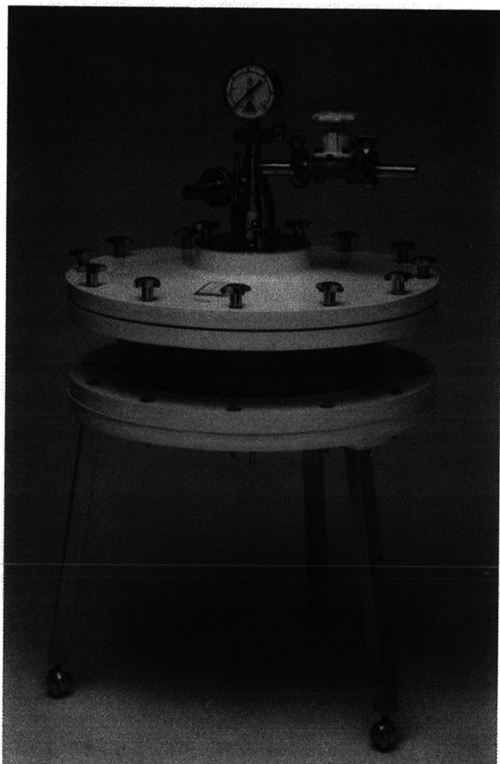


Fig. 19. "Biocompatible" short and wide Whatman column for cellulosic and other "soft" media.

choosing this column type may be that the particles needed can not withstand high pressure and can therefore be used only in short beds. Higher capacity can be obtained only by increasing the column I.D. which again leads to the "pancake waffle type".

CONCLUSION

Recent years or even months have seen spectacular new developments in preparative LC column technology. The field is growing very rapidly. The array of larger systems for process-production preparative LC is already impressive and is bound to increase.

Columns are dry-, slurry- or compression-packed. Some systems involving compression packing do not use compression during the actual chromatography. Whether this is needed or not depends on the difficulty of the problem. For easy separations which can be accomplished in the normal-phase mode, and with relatively large particles, compression may not be needed, or only intermittently. A compressed system can, of course, accomplish both easy and difficult separations, but its cost is much higher.

Considering the large number of manufacturers who all use more or less the same principles, the patent problem is serious. This too could have an influence on the future of some systems.

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CHROMSYMP. 1407

PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES ON A NEW REVERSED-PHASE PACKING MATERIAL, KROMASIL™ C_{18}

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SUMMARY

Preparative reversed-phase high-performance liquid chromatography has found wide use in the production of peptides for pharmaceutical formulations. Purity of the substance and overall economy of the chromatographic system are the most important criterias. In this sense optimized, silica particles and production process with capability to separately control parameters important to chromatography, are essential to high-performance chromatography. Kromasil™ C_{18} packing material was tested and evaluated in respect of its selectivity, flow and pressure properties, resolution, load capacity, recovery, adsorption effects, mechanical strength and chemical degradation.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) of unprotected peptides for their identification and analysis was introduced in the mid 1970s by several authors[1–4]. As a result of the rapid progress over the past 10 years, RP-HPLC has become a valuable method for the preparation of production quantities of biologically active substances as peptides and proteins. Optimized silica packings have made the most important contribution to column efficiency in HPLC. In the production of Kromasil™, a new high-performance silica for liquid chromatography[5], efforts have been focused on surface chemistry, chemical purity, chemical and mechanical stability, pore size and pore-size distribution, surface area, pore volume and particle form and size. The optimization of these parameters together with the proper choice of the mobile phase are essential for separating power and load capacity, long-term stability, high recovery and prevention of denaturation of sensitive biomolecules as proteins.

The main impurities arising from the synthesis of neurohypophysial hormones, such as vasopressin and oxytocin and their analogues, are dimers/polymers and closely related peptides such as deamidated products and isomers. Some of these impurities can be removed by conventional gel and ion-exchange chromatography[6], but for isomers a more selective method such as reversed-phase chromatography is necessary.

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TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF KROMASIL C_{18}

Property measured	*Value*	*Technique*
Particle shape	Spherical	Electron microscopy
Particle size (mean volume)	12.5 μm	Optical microscopy
Particle-size distribution	d_p^{90}/d_p^{10} 1.65	Optical microscopy
Surface area	360 m^2/g	Gas sorption[7]
Pore size (mean)	110 Å	Gas sorption[7]
Pore volume	1.0 ml/g	Gas sorption[7]
Carbon coverage C_{18} monolayer	3.1 $\mu mol/m^2$	Elemental analysis
Endcapped	Yes	
Trace metal content	Na 20 ppm Al <30 ppm Fe 11 ppm	Atomic absorption spectroscopy

MATERIALS AND METHODS

Kromasil C_{18}, 12.5 μm (EKA Nobel, Surte, Sweden) having the physical and chemical characteristics shown in Table I, was slurry-packed into a 250 mm × 10 mm stainless-steel column. Lichroprep C_{18}, 5–20 μm (Merck, Darmstadt, F.R.G.), was packed in the same way. Surface areas and pore-size distributions were measured by the conventional Brunauer–Emmett–Teller (BET) method[7]. The instrumentation consisted of a Digisorb 2600 (Micromeritics, Norcross, GA, U.S.A.). The vasopressin and oxytocin peptides, 8-Arg-vasotocin (AVT), 8-Arg-vasopressin (AVP), d-4-Asn-8-D-Arg-vasopressin (4-Asn-dDAVP), d-8-D-Arg-vasopressin (dDAVP) and oxytocin (OT) were synthesized by fragment condensation (Ferring Pharmaceuticals, Malmö, Sweden). The pumping system was a Waters 600 gradient module with a WISP

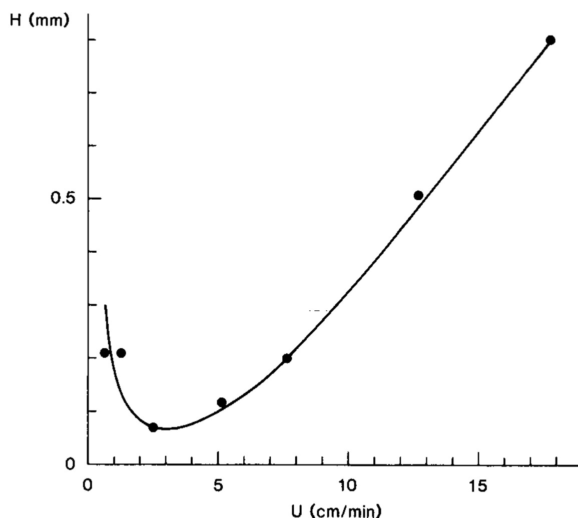

Fig. 1. Dependence of band broadening on flow-rate. Support: Kromasil C_{18} (12.5 μm). Solvent system: acetonitrile–0.1% aqueous trifluoroacetic acid (TFA) (1:4).

autoinjector and a Waters Lambda 410 UV detector (Waters Assoc., Milford, MA, U.S.A.). The solvents were of HPLC grade, filtered through a 0.45-μm filter and sparged with helium. The flow-rate was 4 ml/min (5.1 cm/min) (Fig. 1). Chromatography was performed at ambient temperature.

RESULTS AND DISCUSSION

Resolution and selectivity

As test material for the chromatographic evaluation, a crude preparation of d-8-D-Arg-vasopressin (dDAVP) was chosen (Fig. 2). Apart from dDAVP, B (k' 5.9), it also contained an impurity, A (k' 4.7), arising from the synthesis. The impurity could not be removed by either gel or ion-exchange chromatography. The resolution, R_s, of the two peaks, A and B (Fig. 2), was plotted against the linear flow-rate (Fig. 3). The negative effect of bandbroadening caused by diffusion along the column in this system is reduced by the mass transfer in the stationary phase and therefore the R_s value increases as the flow-rate decreases. Spherical particles, in combination with a narrow size distribution and an high mechanical strength, result in low back pressure in the column (Fig. 3).

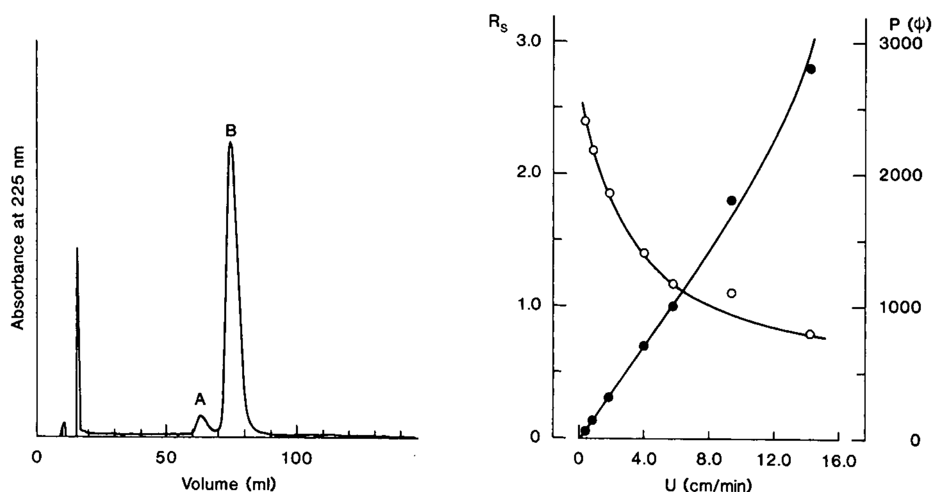

Fig. 2. Resolution of d-8-D-Arg-vasopressin (dDAVP) (B) and an impurity (A). Support: Kromasil C_{18} (12.5 μm). Solvent system: acetonitrile–0.1% aqueous TFA (1:4). The same selectivity was observed on the same support with 0.1 M ammonium acetate as the eluent.

Fig. 3. Resolution, R_s, of peaks A and B (Fig. 2) *versus* linear flow rate, U (○—○) and the pressure drop, ψ, *versus* linear flow rate (●—●). Support: Kromasil C_{18} (12.5 μm).

The influence of the sample volume injected on the theoretical number of plates is given in Fig. 4. A normal sample volume in preparative HPLC is 2–5% of the column volume, but this depends on the sample, the selectivity of the compounds to be separated and the theoretical number of plates needed to separate a given sample. In the example (α = 1.25 on Kromasil C_{18}), the sample can be dissolved in 75% of the column volume and still show the desired selectivity. Compared to a commonly used

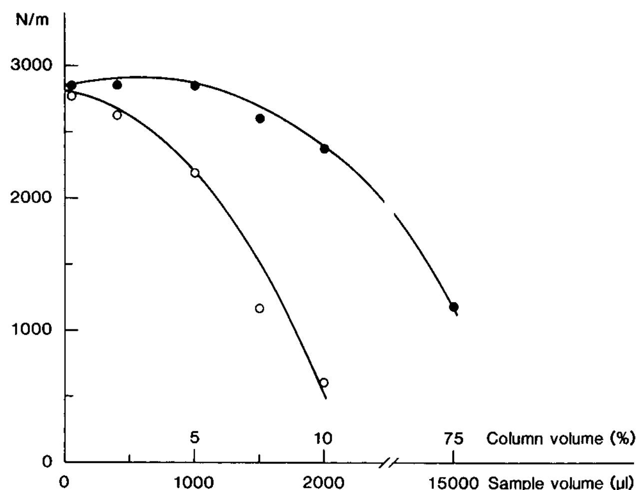

Fig. 4. The number of theoretical plates, N (m), *versus* sample volume or % of column volumes for Kromasil C_{18} (12.5 μm) (●—●) and Lichroprep C_{18} (5–20 μm) (O—O).

preparative RP material, Lichroprep C_{18}, Kromasil C_{18} is not so strongly influenced by the volume injected. The reason for this is the high specific surface area for Kromasil C_{18} and the high degree of carbon coverage. Also considering the amount in mg injected, the Kromasil C_{18} material shows an higher dynamic capacity than that of Lichroprep C_{18} (Fig. 5).

Selectivity

In the selectivity of five different vasopressin and oxytocin peptides, AVT, AVP, 4-Asn-dDVAP, dDAVP and OT, Kromasil C_{18} shows excellent performance and

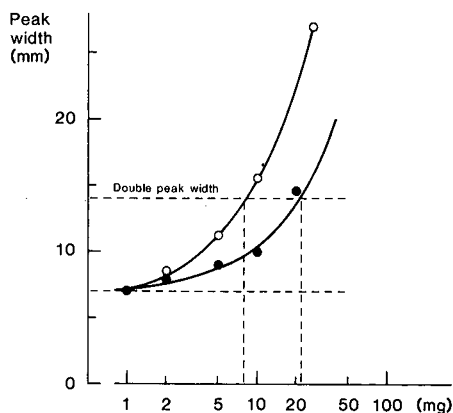

Fig. 5. Evaluation of the dynamic capacity of Kromasil C_{18} (12.5 μm) (●—●) and Lichroprep C_{18} (5–20 μm) (O—O). Solvent system: acetonitrile–0.1 M ammonium acetate (1:4).

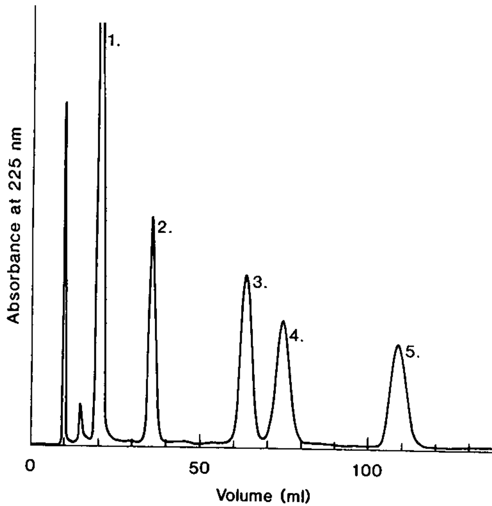

Fig. 6. Elution curve of a mixture of five nonapeptides: 8-Arg-vasotocin (1); 8-Arg-vasopressin (2); d-4-Asn-8-D-vasopressin (3); d-8-D-Arg-vasopressin (4) and oxytocin (5). Support: Kromasil C_{18} (12.5 μm). Solvent system: acetonitrile–phosphate buffer (pH 7.0) (18:82, v/v).

peak asymmetry factors in the range of 0.99–1.10 (Fig. 6). The Lichroprep C_{18} column cannot totally separate the same mixture, especially 4-Asn-dDAVP from dDAVP, and the asymmetry factor 1.0–2.0 indicates a tailing effect for the more basic peptides AVT and AVP (Fig. 7). Another example of the difference in resolving capacity is shown in Fig. 8. The more hydrophobic cyclic tetradecapeptide somatostatin was eluted with a k' value of 2.3 from Kromasil C_{18} (Fig. 8A), while k' increases to 5.7 on Lichroprep

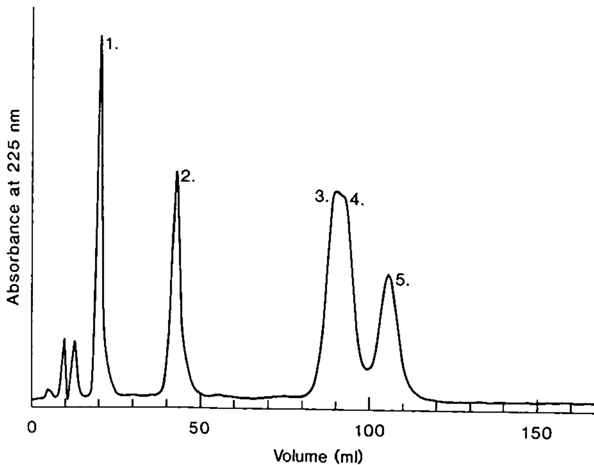

Fig. 7. Elution curve of a mixture of five nonapeptides as in Fig. 6. Support: Lichroprep C_{18} (5–20 μm). All conditions as in Fig. 6.

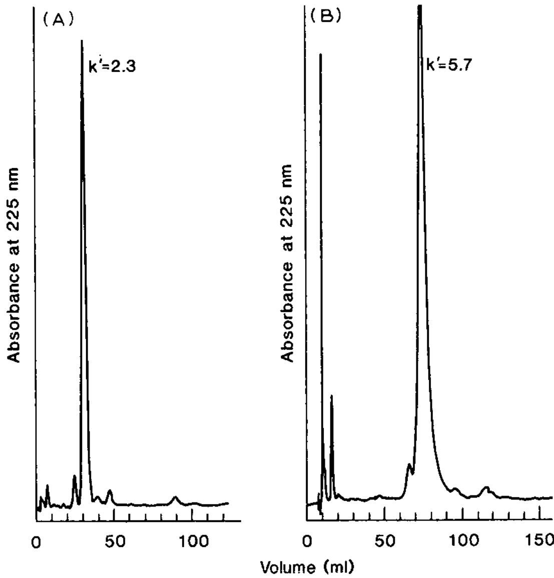

Fig. 8. Chromatography of somatostatin on Kromasil C_{18} (12.5 μm) (A) and Lichroprep C_{18} (5–20 μm) (B). Solvent system: acetonitrile–0.1 *M* ammonium acetate (30:70, v/v).

C_{18} and the chromatogram shows an extremely long tail and a decreased selectivity between the impurities and the main peak (Fig. 8B).

Undesired interaction between the stationary phase and the solute is a common

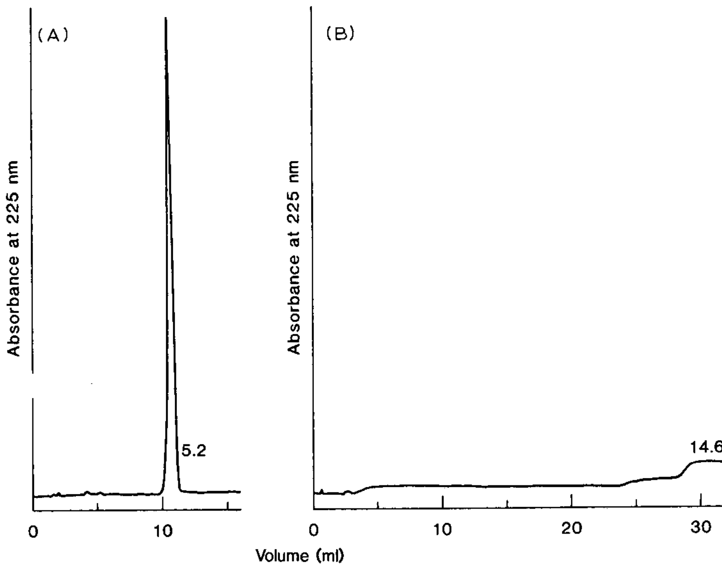

Fig. 9. Chromatography of N,N-diethylaniline on Kromasil C_{18} (A) and Lichroprep C_{18} (B). Chromatographic conditions: columns 200 mm × 4.6 mm; solvent system acetonitrile–water (70:30, v/v); flow-rate 2 ml/min.

problem in liquid chromatography. This interaction is probably due to residual silanols or other adsorption sites, *e.g.*, traces of metals[8,9]. The chemical purity of the silica matrix and high carbon coverage contribute to minimizing these effects. This can be seen by chromatographic a basic substance such as N,N-diethylaniline (Fig. 9). Adsorption of the basic compound was tested on Kromasil C_{18} and Lichroprep C_{18}. The N,N-diethylaniline was easily eluted from the Kromasil column, after the first injection (20 nmol) (Fig. 9A), but was totally adsorbed on the Lichroprep column, and after seven injections each of 20 nmol the aniline appeared as a broad hill (Fig. 9B).

The same difference in retention and selectivity was observed with the Arg-containing peptide, dDAVP (Fig. 10). The columns were washed with ten column volumes of 0.1% aqueous trifluoroacetic acid (TFA) before equilibration in the mobile phase. On Lichroprep C_{18} the peptide was strongly retained and gave rise to a distorted peak and an asymmetry factor of 3.6, without separation of the main peak from the impurity. However, the peptide was eluted normally from Kromasil C_{18} with an α value of 1.45.

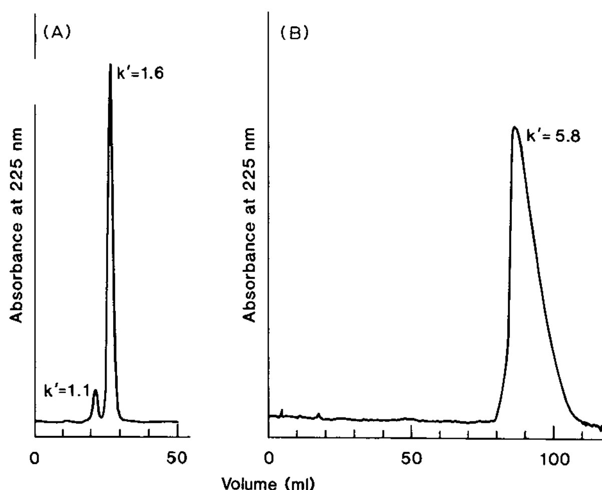

Fig. 10. Chromatography of dDAVP on Kromasil C_{18} (A) and Lichroprep C_{18} (B) after washing with 0.1% aqueous TFA (ten column volumes) and equilibration in acetonitrile–0.1% aqueous TFA (1:4, v/v).

Degradation

An important parameter in preparative RP-HPLC is the chemical and mechanical stability of the stationary phase. Since 0.1% aqueous TFA is a common eluent for the separation of peptides and proteins[10] it was of interest to determine the chemical stability under this condition. A recent investigation on C_1–C_4 organic ligands shows that there is a significant loss of bonded phase during elution with 0.1% aqueous TFA[11]. The organic material degraded under this condition or traces of metals eluted from the column may contaminate the active substance and cause denaturation and undesired effects[12]. The degradation of three different C_{18} bonded phase packings, Kromasil C_{18}, Lichroprep C_{18}, 5–20 μm, and Nucleosil 100-5, C_{18},

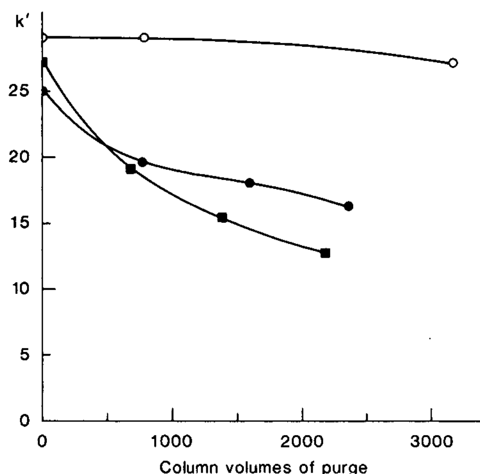

Fig. 11. Degradation of C-bonded phase packings after purging with 0.1% aqueous TFA at 70°C. ○—○, Kromasil C_{18}; ●—●, Nucleosil C_{18} and ■—■, Lichroprep C_{18}.

was investigated under accelerated conditions. The columns (200 mm × 4.6 mm) were purged with 0.1% aqueous TFA at 70°C, at a flow-rate of 2 ml/min. The capacity factor, k', for butylbenzene was determined. The purging process and the capacity factor measurement was repeated and curves showing k' *versus* column volumes were plotted as a measure of the hydrolytic stability (Fig. 11).

Mechanical stability

The mechanical stability of the stationary phase depends only on the naked silica itself. In analytical HPLC systems, the pumps work under constant pressure/flow

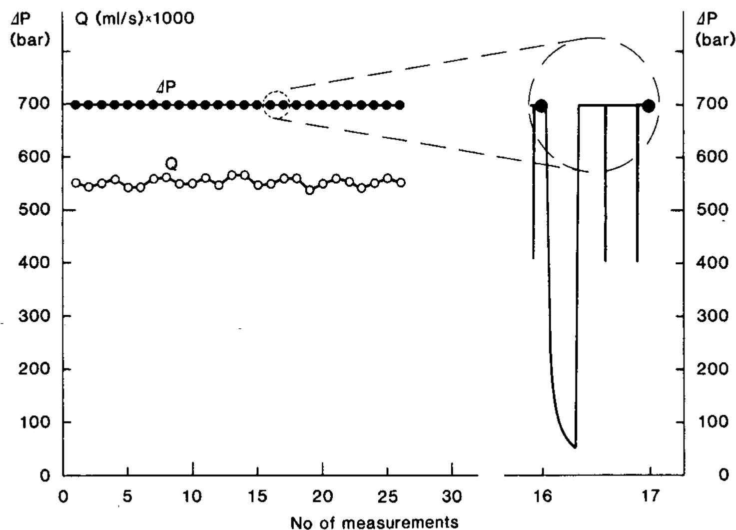

Fig. 12. Mechanical stability of unmodified Kromasil. The flow-rate, Q, was plotted *versus* the pressure drop along the column, ΔP. For further details see the text.

conditions. At the moment of injection, the pressure is drastically decreased, and this is followed by a rapid increase to the preselected level. In our investigation we reproduced this performance. A stainless-steel column (250 mm × 4.6 mm) was slurry-packed with 8-μm unmodified Kromasil and kept under constant pressure (700 bar). Every minute, the pressure was momentarily decreased to 20–50 bar, followed by an increase to 700 bar. The flow-rate was measured and plotted (Fig. 12). This procedure was repeated 26 times. After that, the column was emptied and the stationary phase was examined under a microscope (× 200 enlargement). None of the testing procedures indicated that any fines had been produced.

The change in chromatographic behaviour of Kromasil C_{18} over time under normal conditions was also investigated. Acetonitrile–0.1 *M* ammonium acetate (1:4) was chosen as the mobile phase system. More than 1400 column volumes passed through, which corresponds to 400 analyses. The selectivity between peaks A and B (Fig. 2) was calculated and plotted. No significant change in selectivity was observed (Fig. 13).

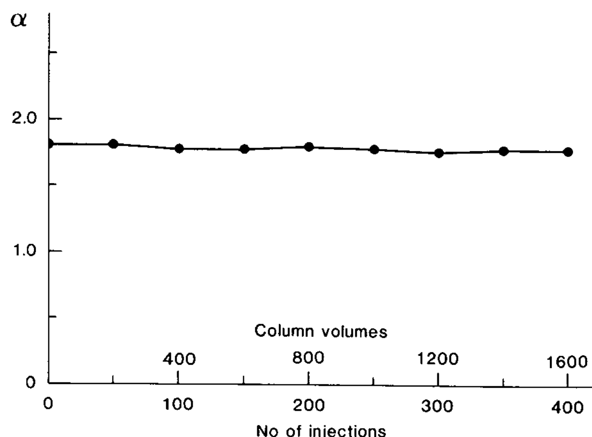

Fig. 13. The change in selectivity, α, of peaks A and B (Fig. 2) plotted *versus* time and column volumes. Support: Kromasil C_{18} (12.5 μm). Solvent system: acetonitrile–0.1 *M* ammonium acetate (1:4).

Recovery

In production-scale chromatography the yield of pure substance per unit time is an important parameter. It has been pointed out that one of the major causes of irreversible interaction in RP-HPLC is the interaction between the solute, the stationary phase and the mobile phase[13]. A 5-mg amount of pure peptide (dDAVP) was injected into the Kromasil C_{18} column under isocratic conditions, and when an increase in the absorbance at 280 nm was registered the eluate was collected manually and the volume measured. The absorbance at 274 nm of the diluted sample was correlated to a known concentration of the peptide dissolved in the mobile phase. The recovery of the peptide chromatographed under acidic (0.1% aqueous TFA) or neutral conditions (0.1 *M* ammonium acetate) was 98 and 95%, respectively. Corresponding figures for Lichroprep C_{18} were 84 and 94%, respectively. When the amount of

organic solvent in the mobile phase was increased no sample was eluted from the Kromasil C_{18} column with acidic or neutral eluents, nor from the Lichroprep C_{18} column with a neutral buffer. However, an increase in the UV signal was observed with the Lichroprep C_{18} column and the acidic 0.1% aqueous TFA.

CONCLUSION

This study has focused on parameters affecting preparative RP-HPLC: selectivity, flow and pressure properties, recovery and chemical and mechanical stability. These parameters are mainly determined by the structure of the naked silica and the method of preparation of chemically modified silica packings. We have shown that under strictly controlled conditions these are the important characteristics which together with a volatile buffer, acidic or neutral, determine whether the proposed purity of the peptide substance can be achieved.

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CHROMSYMP. 1410

ECONOMIC LABORATORY PRACTICE IN PREPARATIVE COLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

For isolating from 1 mg up to 200 g of pure substance from unknown and complex mixtures, a set of materials and methods is presented and evaluated by several applications. The procedure combines automated sequential high-performance liquid chromatography with gradient elution on a 50 mm × 21.4 mm, 3-μm packed column. The method development time is very short and the chromatographic system has an high specific production rate. The preparative performance is discussed, and compared with other approaches.

INTRODUCTION

On the laboratory scale, flexible automation is required in column liquid chromatography to accommodate frequent application changes and variable quantity ranges. Technical optimization is less critical than for industrial-size separations, but economic aspects are seldom negligible. For isolating several grams of pure substance from unknown and complex mixtures, the time is considerably reduced by using a 3-μm packed short column of about 20 mm I.D., supplemented by an automatic system monitoring the repeatability of unattended operations. The same instruments may also be used for analytical purposes and in a research environment. A method is presented with practical details and several applications are described. The performance is discussed, and compared with other approaches.

Preparative column liquid chromatography (PCLC) may be classified according to the order of magnitude into four groups, as shown in Table I. Micropreparative chromatography isolates subnanomole quantities of proteins and polypeptides for microsequence analysis[1,2], while macropreparative fractionation may reach kilogram levels. This upper limit corresponds to a maximum column diameter of about 200–300 mm, and a maximum flow-rate of about 5 l/min. At the other end of the range, the minimum preparative sizes are continuously being lowered, just as upper limits are rising in industrial applications. The experiments reported here exclude micro-preparative work and do not exceed 200 g of collected mass, column diameter of 41.4 mm and flow-rates of 50 ml/min.

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TABLE I

SIZE CLASSIFICATION OF LABORATORY-SCALE PCLC

Collected mass (g)	*Designation*	*Column diameter (mm)*	*Purpose*
$<10^{-6}$	Micropreparative	<2	Bioanalysis
10^{-6}–10^{-3}	Semipreparative	2–10	Chemical analysis
10^{-3}–1	Preparative	10–50	Identification and reactions
1–10^{3}	Macropreparative	20–300	Manufacture of standards

EXPERIMENTAL

Materials

The chromatograph used was a binary-gradient, automatic, preparative system (Gilson Medical Electronics, Villiers le Bel, France). It consisted of two elution pumps (303 with a 25- or 50-ml/min pump head), injection pump (302 with a 10-ml/min pump head) and a manual injection valve with 0.5-ml loop (7125; Rheodyne, Cotati, CA, U.S.A.), a manometric module (803 C), dynamic mixer (811 A), UV-absorbance detector with 0.2-mm lightpath cell (116), a fraction collector with a three-port valve (202 C), system controller (IBM AT with hard disk, EGA graphic card, mouse, MS DOS 3.1 and Windows software), contact module (506), system software (714) and printer (Hewlett-Packard Thinkjet). This equipment is characterized by a modular hydraulic structure and programmable operating functions.

All columns were Dynamax (Rainin Instrument, Woburn, MA, U.S.A.), from 4.6 to 41.4 mm I.D., cartridge-type replaceable columns and guard modules, with permanent, axial-compression end-fittings and replaceable inlet filter. The method described is based on the direct use, as a separation column, of a 50 mm × 21.4 mm guard module, packed with 3-μm stationary phase, equipped with a 0.5-μm inlet filter and installed inside a permanent holder. Solvents, from various sources, were either of high-performance liquid chromatography (HPLC) grade or distilled, according to the application, and always filtered through 0.5-μm filters.

Method

The method was developed to isolate several grams of pure substances from unknown and complex mixtures. In this type of separation problem, the components which have already been identified are rare. Generally, only some indications of the chemical structure and functional groups are available. From this limited information, the chromatographer has to choose the separation phase system as well as the detection conditions. The procedure is based on the direct use of the 50 mm × 21.4 mm, 3-μm column, systematically under gradient elution. Such a column contains about 9 g of silica-based stationary phase and the total volume of the mobile phase inside is about 11 ml. Most mixtures were resolved on a reversed-phase octadecylsilane (ODS) column with water–methanol at ambient temperature. Under these conditions, the maximum pressure seldom exceeds 8 MPa (80 bar, 1100 p.s.i.) for a typical flow-rate of 15 ml/min. Two detection channels were generally used, either at two

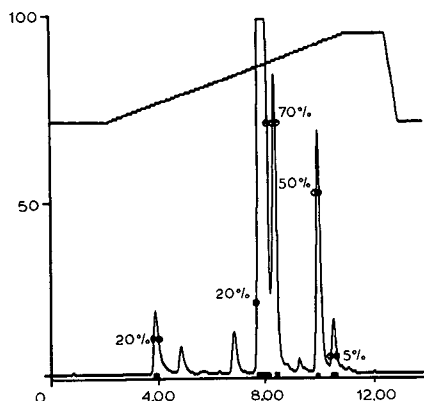

Fig. 1. Separation of phenolic esters. For details, see Table II, No. 3; detection, 300 nm, 10 a.u.f.s. Time scale in min. Signal and gradient profile in %.

different wavelengths (from 190 to 380 nm) or at two different sensitivities (from 0.025 to 50 a.u.f.s., related to a 10-mm lightpath). The unknown sample (solid or concentrated liquid mixture) was dissolved in a minimum volume of mobile phase having an elution strength equal to or below that of the initial gradient composition, in order to generate favourable effects of enrichment or peak compression. It was then filtered through a 0.5-μm filter. This sample solution had a typical concentration of 10 mg/ml. No other sample pretreatment was performed in the cases described.

The procedure included three basic steps. (1) Determination of the mobile phase flow-rate and gradient profile by manual sample injections of 0.5 ml. (2) Determination of the injection volume by automatic injections of increasing volumes, approaching volume or mass overload, but generally not higher than the column linear capacity threshold. This volume reached 5 ml in favourable cases. (3) Unattended, multicycle, preparative separation, with fraction collection combining time and signal criteria. When the objective was to purify one or a few major components, as opposed to isolating trace components or impurities, the last step was preceded by a tentative optimization of fractionation cut points, based on a compromise between purity and recovery. Variable fractionation points were selected from the chromatogram displayed on the screen, as illustrated by Fig. 1. The instrument design also allowed us to choose the most appropriate collection vessels, generally 250-ml round-bottom flasks. Purity tests of collected fractions were directly performed by manual injections of 0.5 ml of the collected solutions, on the same column and under a 10- to 100-fold higher detection sensitivity.

RESULTS AND DISCUSSION

These methods have been extensively applied to isolate from 1 mg to 200 g of pure substances from natural products, plant protective agents, pharmaceutical compounds, synthetic oligopeptides and metal complexes. Some of these applications are presented in Table II (separations 1–9 on column 1) where they can be compared with others, performed on columns of closely related, as well as very different, dimensions.

TABLE II

PCLC SEPARATIONS ON ODS COLUMNS BY GRADIENT ELUTION WITH WATER AND METHANOL

Exceptions: 9 and 16, water and acetonitrile on ODS (with 0.1% tetrahydrofuran for 9); 7, *n*-hexane and dichloromethane on silica; 13, *n*-hexane and methyl *tert.*-butyl ether on silica; 14 and 15, methyl *tert.*-butyl ether and methanol on silica; 17 and 18, potassium phosphate (pH 6.0) from 5 to 300 m*M* on Dynamax AX (anion exchanger).

No.	*Application*	*Flow-rate (ml/min)*	*Injected volume (ml)*	*Injected mass (mg)*	*Cycle time (min)*	*Throughput (mg/min)*	*Specific throughput (mg/g · l · h)*	*No. of compounds collected/detected*
Column 1: 50 mm × 21.4 mm, 3 μm, 100 Å (*ca.* 3000 theoretical plates)								
1	A: Lavender oil	15	2	20	15	1.3	40	5/12
2	B: Peppermint oil	15	5	125	10	12.5	560	3/16
3	C: Phenolic esters	10	1	10	13	0.8	30	5/14
4	D: Triptycenes	15	2.5	25	12	2.1	62	5/18
5	E: Analgesic	10	2	120	20	6.0	200	3/24
6	F: Iron complexes	10	0.5	5	10	0.5	33	4/16
7	F: Iron complexes	15	1	12	15	0.8	24	5/21
8	G: Cerebrosides	20	1	25	10	2.5	83	10/19
9	H: Oligopeptides	20	1	25	14	1.8	43	5/41
Column 2: 100 mm × 21.4 mm, 3 μm, 100 Å (*ca.* 8300 theoretical plates)								
10	I: Camomile oil	15	5	50	30	1.7	12	3/40
11	J: Perfume	15	5	50	40	1.2	7	6/44
12	K: Eucalyptus oil	15	0.5	25	30	0.8	6	8/12
Column 3: 50 mm × 21.4 mm, 8 μm, 60 Å (*ca.* 1600 theoretical plates)								
13	L: Insecticide	24	0.2	250	14	17.9	355	1/8
Column 4: (50 + 250) mm × 21.4 mm, 8 μm, 60 Å (*ca.* 9000 theoretical plates)								
14	M: Pesticide	24	0.4	120	20	6.0	14	1/7
15	N: Insecticide	24	1.5	110	18	6.1	16	2/2
16	O: Fungicide	24	1.5	75	72	1.0	1	9/17
Column 5: 250 mm × 41.4 mm, 12 μm, 300 Å (*ca.* 5000 theoretical plates)								
17	P: Lactoglobulins	49	30	1300	45	28.9	5	4/8
18	Q: α-Lactalbumin	49	40	2000	65	30.8	3	1/32

Preparative performance

The results of preparative separations are currently expressed in terms of the throughput (TP) and production rate (PR). This second parameter is of prime importance for the industrial scale, where one is dealing with known mixtures. The throughput, *i.e.*, injected sample mass divided by separation cycle time, varies from 0.5 to 12.5 mg/min for separations 1–9. It is mainly a function of the sample solubility (10–60 mg/ml), number of compounds detected (12–41), and number of compounds collected (3–10). However, neither TP nor PR evaluates the chromatographic performance. Table II indicates, as a criterion of preparative performance, the specific throughput (STP), defined as the throughput divided by the mass of stationary phase and by the volume of mobile phase

$$\mathrm{STP} = 4m_s/\pi d_c^2 L\rho F t^2$$

where m_s is the sample mass injected, d_c and L the column inner diameter and length,

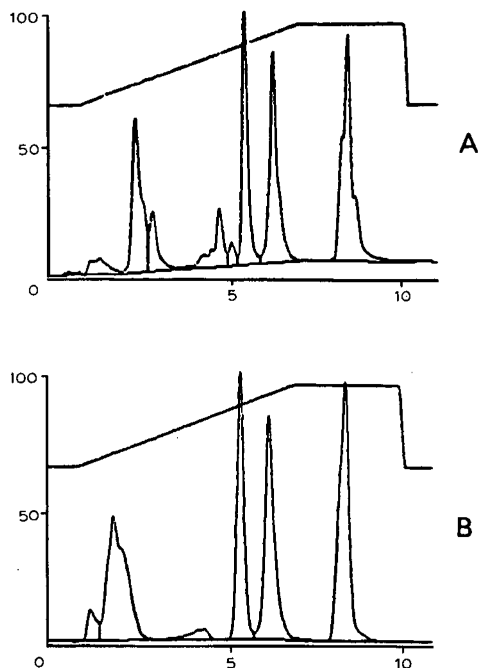

Fig. 2. Loadability test of peppermint oil. For details, see Table II, no. 2. (A) Concentration of injected solution is 1% of oil in 60% aqueous methanol; 0.5 ml, containing 5 mg, are injected and detected at 230 nm, 2 a.u.f.s. (B) Concentration of injected solution is 2.5% of oil in 60% aqueous methanol (emulsion); 5 ml, containing 125 mg, are injected and detected at 230 nm, 30 a.u.f.s. Time scale in min. Signal and gradient profile in %.

ρ the stationary-phase packing density (0.5 g/ml, assumed to be constant in Table II), F the mobile phase flow-rate, and t the separation cycle time. STP is thus the sample load of the phase system per time unit. It is much higher for experiments 1–9 than for the other experiments reported in Table II, except for 13, which will be discussed further. This index has been already used[3] to demonstrate the benefits of the recycling technique[4] compared with single-pass separations. At that time, the best value was only 5 mg/g · l · h, obtained for the separation of two isomers, from 2.3 g, in 1 h, on a 500 mm × 32 mm, 11-μm column, with mass overload and five-cycle recycling. Today, in the case of experiment 2, STP is more than 100 times higher. Two extreme chromatograms, performed as loadability tests for this separation, are shown in Fig. 2. Similarly, the production rate of a compound, *i.e.*, collected fraction mass, m_f, divided by separation cycle time, can be related to the amounts of chromatographic phases and is called the specific production rate (SPR) for this compound. The corresponding expression is

$$\mathrm{SPR} = 4m_f/\pi d_c^2 L\rho F t^2 n$$

where n is the number of injections corresponding to m_f. SPR is 10 mg/g · l · h for experiment 15 which produced 50 g of pure insecticide in 700 cycles with a yield of 70%. It is more than 200 mg/g · l · h for experiment 13 which produced 190 g of another

insecticide, purified from 76 to 99%, in 1200 cycles, *i.e.*, 12 days. This second example is considered as being the ultimate in productivity. The mobile phase composition and flow-rate were optimized, and the liquid sample was injected without being previously dissolved. At the other extreme, experiment 9 produced about 1 mg of each collected oligopeptide in 25 min.

Benefits of a short column, packed with fine particles

Already popular as "fast" HPLC on 4- to 5-mm diameter columns, the advantages of 3-μm packed, short columns are especially appropriate for PCLC, featuring short separations times (10–20 min), low solvent consumption (100–200 ml per cycle on a 21.4-mm diameter column) and high solute concentration. Moreover, the substance concentration is further increased by gradient elution, and gradient elution benefits from rapid column reconditioning (2–3 min). A solvent change requires about 5 min. With silica, three preliminary cycles are enough to stabilize the system. In addition, the fact that up to 200 g can be purified with only 9 g of stationary phase may justify the use of very selective and expensive phases, especially in affinity chromatography, for producing antibodies and enzymes.

Other benefits concern method development and method transfer. With the above-described procedure, the time needed for method development was 3–5 h, with typically 15–20 injections, including 10 manual ones. If greater efficiency is needed, transfer from column 1 to column 2 or 4 is also very rapid, usually limited to a proportional adaptation of the time points of the gradient profile to the length and flow-rate of the new column. If an higher throughput is desired, transfer from column 1 to column 5 should solve the problem. It would require only a change of pump head on the two elution pumps. Experiment 13 shows that a very satisfactory result is achieved on column 3, having 8-μm instead of 3-μm particles.

Fig. 3 presents Van Deemter curves obtained with preparative injections into

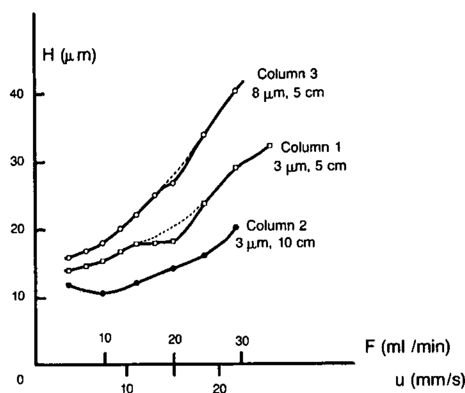

Fig. 3. Plate height (H) *versus* linear velocity (u). Columns: 1 = Rainin, 50 mm × 21.4 mm, 3-μm Microsorb C_{18}, spherical, 100 Å; 2 = Rainin, 100 mm × 21.4 mm, 3-μm Microsorb C_{18}, spherical, 100 Å; 3 = Rainin, 50 mm × 21.4 mm, 8-μm Dynamax C_{18}, irregular, 60 Å. Mobile phase; 80% aqueous methanol, isocratic; injected volume, 0.5 ml; sample, test mixture, containing 0.01 g/l uracil, 0.02 g/l acetophenone, 0.40 g/l anisole and 1.13 g/l toluol, dissolved in mobile phase; reference peak, toluol (k' = 1.6 on columns 1 and 2, 2.0 on column 3); detection, 254 nm, 0.2 a.u.f.s. F = Flow-rate.

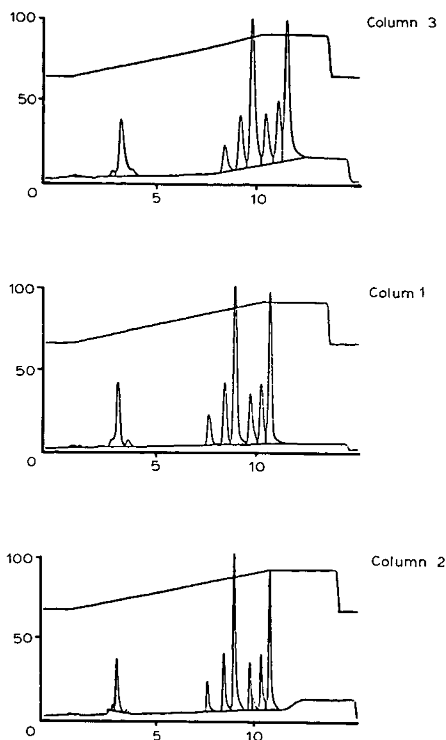

Fig. 4. Column comparison. Columns 1–3 as in Fig. 3; mobile phase, gradient from 65 to 90% of methanol in water; flow-rate, 15 ml/min; injected sample, 0.5 ml, containing 25 mg of eucalyptus oil; detection, 230 nm, 3 a.u.f.s. Time scale in min. Signal and gradient profile in %.

columns 1, 2 and 3 (Table II). The distortions of curves 1 and 3 may be attributed to temperature effects, as mentioned by Verzele and Dewaele[5], and also to the column technology, since column 2, in contrast with the other two, is not of the cartridge type with an holder, *i.e.*, it has a single instead of a double wall. These three columns have been compared in the separation of eucalyptus oil (Fig. 4). This showed that column 1 is preferred. Other chromatograms are presented in Figs. 5 and 6.

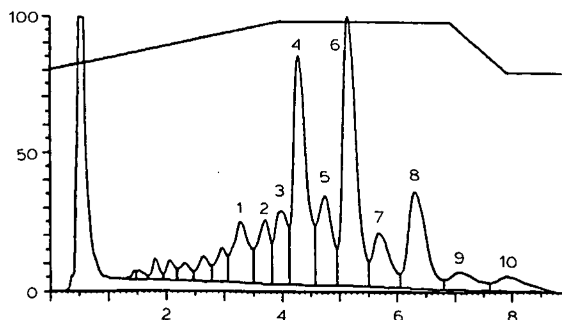

Fig. 5. Separation of cerebrosides. For details, see Table II, No. 8; detection, 230 nm, 1 a.u.f.s.; a silica thin-layer chromatogram of this sample revealed only three spots. Time scale in min. Signal and gradient profile in %.

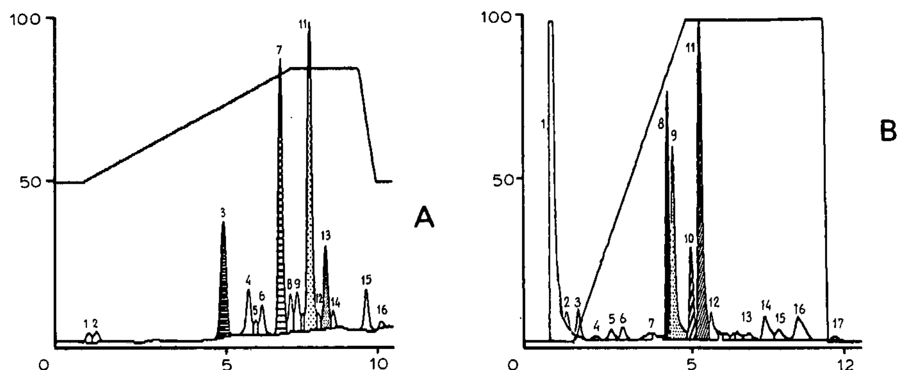

Fig. 6. Separation of iron complexes. (A) For details, see Table II, No. 6; detection, 254 nm, 0.1 a.u.f.s. (B) For details, see Table II, No. 7; detection, 254 nm, 0.5 a.u.f.s. Time scale in min. Signal and gradient profile in %.

Automation

After method development (1/2 day) the chromatograph operates unattended, generally during the other half day for 1 g, and up to *ca.* 1 week for up to 100–200 g. In order to operate for 168 h a week instead of just 40 h, the instrument requires several fail-safe features and an high level of repeatability. The requirements for the fraction collector have already been examined in detail[6]. The model chosen is capable of interrupting the system in the case of retention shifts and baseline drifts, in accordance with continuously adjustable parameters. It also has provision for flushing the module with an inert gas. The system software can activate a predefined emergency programme from a contact closure in case of failure or a pressure outside of predefined limits. The column is unbreakable. There is no need to degas the solvents, the pump heads are not sensitive to air-bubbles (the high-pressure mixing eliminates them), and

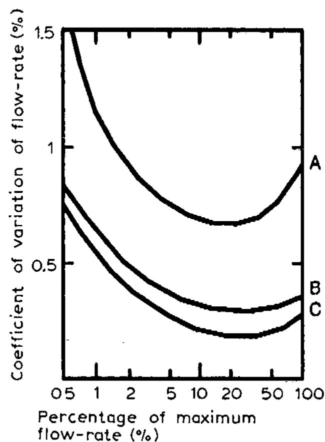

Fig. 7. Precision of Gilson piston pumps. (A) Chloroform (viscosity, 0.57 cP at 20°C); (B) water (1.0 cP at 20°C); (C) 60% aqueous methanol (1.8 cP at 20°C). Measurements were made by volumetry, gravimetry and chromatography from retention times. Each curve was drawn from 21 points and each point represented from 5 to 50 measurements. The deviation of experimental points from each curve was less than 25%. These curves are valid for all models of interchangeable pump heads, at ambient temperatures and under any positive pressure below the specified limit.

the fraction collector is able to neglect possible air spikes thanks to an adjustable time-constant filter.

Lastly, the repeatability of each cycle mainly depends on the pumping system. Fig. 7 shows precision curves, obtained with the pumps used for the experiments described. Repeatability of the flow-rate as well as of the injected volume depends both on the nature of the solvents and on the flow-rate selected. The column is very stable over 1000 cycles. Loss of efficiency was seldom observed and generally well compensated for by hand-tightening of the axial compression fittings.

CONCLUSION

As a solution for isolating from 1 mg to 200 g of pure substances from unknown and complex mixtures, the procedure presented implements automated sequential HPLC with gradient elution from a 50 mm × 21.4 mm, 3-μm column. This small column has an outstandingly high production rate. Having completed method development, the chromatographer can leave the instrument to operate unattended for up to 10 days. The same instrument is used to check the purity of collected fractions and is compatible with current-scale analytical HPLC. The column cost, solvent consumption, labour and investment are considerably lower with such a procedure than with manual injections into long columns, packed with coarser particles. While analytical separation is a measuring process, preparative separation is in essence a measured operation, where economy factors are meaningful at any scale.

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SEPARATION OF NATURAL PRODUCTS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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SUMMARY

Centrifugal liquid–liquid partition chromatography (CPC) with a Sanki CPC apparatus has been used to perform efficient separations of various classes of natural products. Applications involving flavonoids, saponins, coumarins, anthraquinones, phenolic acids and naphthoquinones are described. Some of the examples concern isolation of pure compounds from crude plant extracts. CPC is also compared with other counter-current chromatography techniques.

INTRODUCTION

Centrifugal partition chromatography (CPC) relies on the principle of liquid–liquid partition for the separation and purification of compounds. The technique differs from that of Craig counter-current distribution and droplet counter-current chromatography (DCCC)[1] in the application of a centrifugal force in the course of separation, which results in time saving. CPC or centrifugal counter-current chromatography (CCCC), as it is also known, is a continuous process of non-equilibrium partition between two immiscible phases contained in rotating coils[2] or cartridges[3]. The cartridge system[3] is a recently introduced technique which has, until now, found relatively little application[4]. Most work has been carried out on tannins[5], but examples of separations also include the purification of labile tunichromes[6] and the isolation of retinals[7]. We report here an extension of the applications of CPC to a diverse range of natural products. Separations with the Sanki cartridge CPC system were performed, *e.g.*, on flavonoids, saponins, coumarins, anthraquinones and naphthoquinones.

Centrifugal partition chromatography does not rely on solvent systems which have to form droplets, as in DCCC. With few exceptions, most solvent mixtures which form two immiscible layers are acceptable. While the choice of solvent system cannot definitely be determined by thin-layer chromatography (TLC), as is the case with DCCC[8], preliminary results indicate that for optimum separations, the R_F of the compounds to be separated should lie in the range 0.2–0.5 when TLC is carried out with the mobile phase of the solvent system on silica gel plates. In most (but not all) cases, a mixture which can be resolved by TLC is also resolved by CPC. In some instances, CPC provides the resolution of a mixture that cannot be resolved by silica gel TLC.

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Representative solvent systems include: chloroform–methanol–water (33:40:27) [for polar substances]; ethyl acetate–94% ethanol–water (2:1:2) [for polar substances]; light petroleum (b.p. 60–95°C)–ethyl acetate–methanol–water (18:42:30:30) [for non-polar substances[9]]; hexane–acetonitrile–methanol (40:25:10) [for non-polar substances]. The choice of these solvents is based on TLC investigations of the particular sample to be separated (to get a first idea of the suitability of a two-phase system), as well as literature references. For example, the system ethyl acetate–94% ethanol–water (2:1:2) has previously proved suitable for the rotation locular counter-current chromatography (RLCC) separation of saponins[10] and is here used for the separation of flavonoid glycosides.

EXPERIMENTAL

All separations were carried out at 20°C on a CPC Model LLN (Sanki Engineering, Kyoto, Japan), connected to a 2238 Uvicord SII detector (254 nm) (LKB, Bromma, Sweden), 600 chart recorder (W + W Scientific, Basle, Switzerland) and an LKB Ultrorac II fraction collector. The continuous-flow centrifuge was fitted with six Type 250W cartridges (total volume 125 ml). Samples were injected by means of a six-way valve and a 3-ml sample loop. Monitoring of fractions was carried out either by UV at 254 nm or TLC on silica gel aluminium-backed plates (Merck, Darmstadt, F.R.G.). The apparatus was first filled with stationary phase and then the mobile phase was pumped through. When elution of the stationary phase was complete and mobile phase exited from the cartridges, the sample was introduced.

With the Sanki CPC Model LLN apparatus, the speed of the centrifuge rotor can be regulated. Higher rotation speeds generally lead to a better resolution of the sample but at the same time increase pumping pressures. Therefore, speeds of 1000–1500 rpm are desirable for carrying out efficient separations at a relatively high flow-rate of mobile phase, keeping analysis times short. The viscosity of the mobile phase is important, however, and for chloroform-containing solvents, rotation speeds and flow-rates have to be diminished in order to avoid pressure build-up. The choice of parameters is thus a rather empirical process, the aim being to increase both the rotation speed and flow-rate (via higher pump speeds) without causing overpressure problems or loss of resolution.

RESULTS

Separation of flavonoid aglycones

Fig. 1 shows the separation of the flavanone hesperetin (1) and the flavonols kaempferol (2) and quercetin (3), with the same solvent system, chloroform–methanol–water (33:40:27) (descending) for (a) RLCC[11], (b) DCCC[11] and (c) CPC. In each case, elution was according to the order of increasing polarity: hesperetin, kaempferol, then quercetin. Whereas DCCC and RLCCC required more than 30 h for complete separation, CPC took only 2.5 h. The solvent consumption for RLCCC was *ca.* 1500 ml, for DCCC *ca.* 550 ml and for CPC *ca.* 300 ml. The low solubility of the flavonoid aglycones limited the sample size in these examples.

The Sanki CPC system offers the possibility to work in the reversed-phase mode. Thus, during separation, the elution mode can be changed, while simultaneously

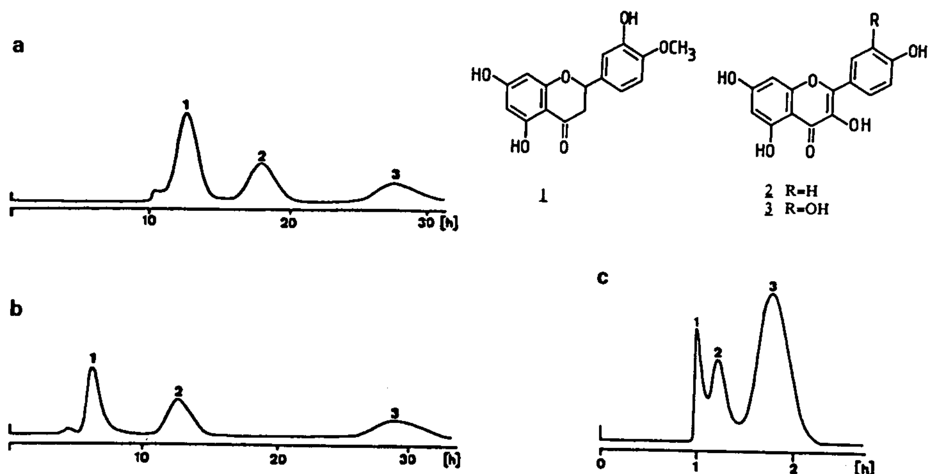

Fig. 1. Counter-current chromatography of hesperetin (1), kaempferol (2) and quercetin (3). Solvent system: chloroform–methanol–water (33:40:27), descending mode. Detection: 254 nm. (a) RLCC separation. Flow-rate, 48 ml/h. (b) DCCC separation. Flow-rate, 18 ml/h. (c) CPC separation. Flow-rate, 2 ml/min. Rotational speed: 600 rpm. Sample: 6 mg in 1 ml of upper phase and 1 ml of lower phase.

changing the phases. An example of this very useful technique is shown in Fig. 2. With chloroform–methanol–water (33:40:27) in the ascending mode, and the aqueous phase as the mobile phase (Fig. 2a), a good separation of the three flavonoid aglycones was obtained. Starting the separation in the ascending mode and then reversing the modes, *i.e.*, changing to the descending mode and eluting with the lower phase of the system, after elution of aglycone 3, produced a much more rapid separation of compounds 1 and 2 (Fig. 2b). However, in this example, aglycone 1 was eluted before aglycone 2.

Separation of flavonoid glycosides

A mixture of rutin (4), hyperoside (5) and quercitrin (6) was resolved by CPC with ethyl acetate–94% ethanol–water (2:1:2) in the descending mode (Fig. 3). The quantity of stationary phase eluted before the appearance of mobile phase (and injection of the sample) was 70 ml. Baseline separation was achieved within 2.5 h,

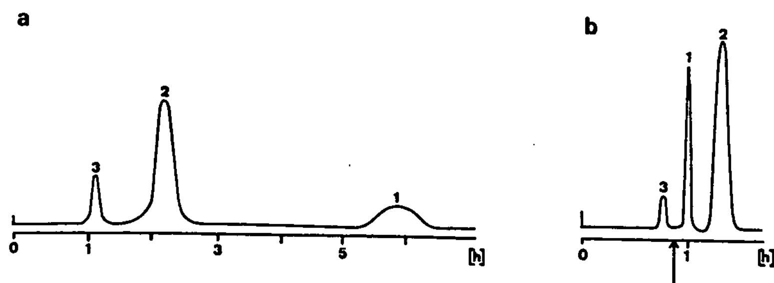

Fig. 2. Separation of hesperetin (1), kaempferol (2) and quercetin (3) by CPC. Conditions as in Fig. 1. (a) Ascending mode. Flow-rate, 1 ml/min. (b) Ascending mode to 55 min. After 55 min, descending mode with the organic phase as the mobile phase. Flow-rate, 1.6 ml/min.

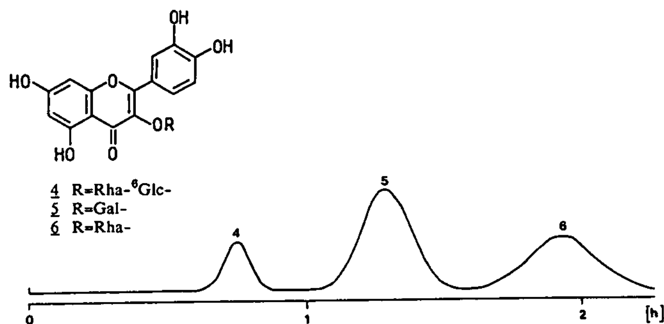

Fig. 3. Separation of rutin (4), hyperoside (5) and quercitrin (6) by CPC. Solvent system: ethyl acetate–94% ethanol–water (2:1:2); descending mode; flow-rate, 3 ml/min. Rotational speed: 1500 rpm. Detection: 254 nm. Sample: 15 mg in 1 ml of upper phase and 1 ml of lower phase. Rha = Rhamnose; Glc = glucose; Gal = galactose.

whereas DCCC separation of the mixture required 9 h[12]. However, direct comparison with the DCCC method was impossible because separation by CPC with the DCCC solvent system chloroform–*n*-butanol–methanol–water (10:1:10:6) (ascending) gave problems of overpressure.

Flavonoid glycosides were separated from sugars and less polar constituents when a methanol extract of *Tephrosia vogelii* (Leguminosae) leaves was subjected to CPC (Fig. 4). There was also partial resolution of rutin (4), isoquercitrin (7) and

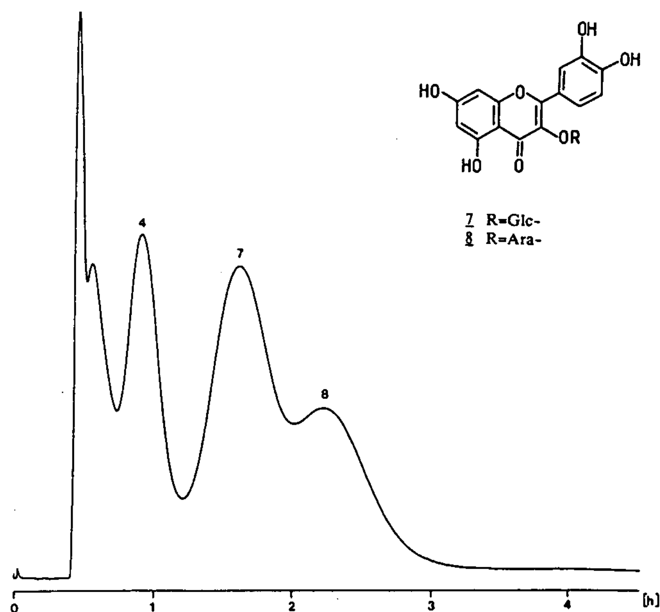

Fig. 4. CPC of a crude extract of *Teprhosia vogelii* leaves. Solvent system as in Fig. 3. Flow-rate: 2.2 ml/min. Rotational speed: 1000 rpm. Detection: 254 nm. Sample: 100 mg of extract in 1 ml of upper phase and 1 ml of lower phase. Ara = Arabinose.

quercetin 3-O-α-L-arabinopyranoside (8) with ethyl acetate–94% ethanol–water (2:1:2) (descending)[13]. It is noteworthy that in both the examples of Figs. 3 and 4, separation of glycosides differing only in the nature of their monosaccharide moieties was achieved.

Separation of triterpene glycosides

Counter-current chromatographic techniques, and especially DCCC, have proved of immense value for the separation of very polar saponins[4]. One example is the isolation of four pure triterpene glycosides from a molluscicidal methanolic extract of *Hedera helix* (Araliaceae) berries by DCCC[8]. The extract was first partitioned between *n*-butanol and water; the molluscicidal *n*-butanol layer was directly subjected to CPC, with the lower layer of a chloroform–methanol–water (7:13:8) mixture as the mobile phase (Fig. 5). The fractions were monitored by TLC. The hederagenin saponins 9–12 were separated within 2 h. Changing the elution mode to ascending after fraction 30, with the upper phase as the mobile phase, enabled two more polar saponins to be eluted in fractions 33 and 35. Fractions 31–34 also contained large quantities of polysaccharide material.

Saponins 9–12 have also been separated by DCCC using the same solvent system[14]. However, this method required 20 h to give the same result.

Separation of coumarins

Attempts to separate the coumarins herniarin (13), umbelliferone (14) and scopoletin (15) with the solvent system chloroform–methanol–water (13:7:8), pre-

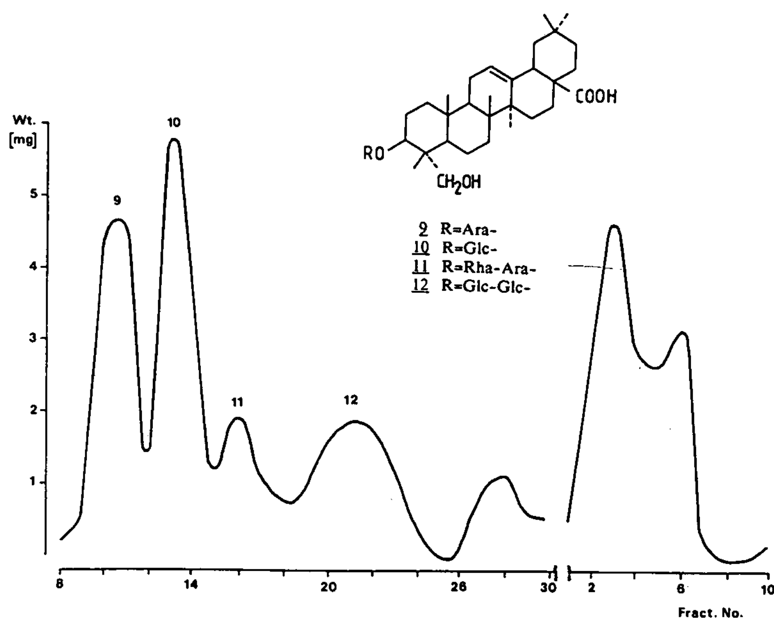

Fig. 5. CPC of a methanol extract of *Hedera helix* berries after *n*-butanol–water partition. Solvent system: chloroform–methanol–water (7:13:8); descending mode; flow-rate, 1.5 ml/min. Rotational speed: 700 rpm. Sample: 100 mg of extract in 1 ml of lower phase.

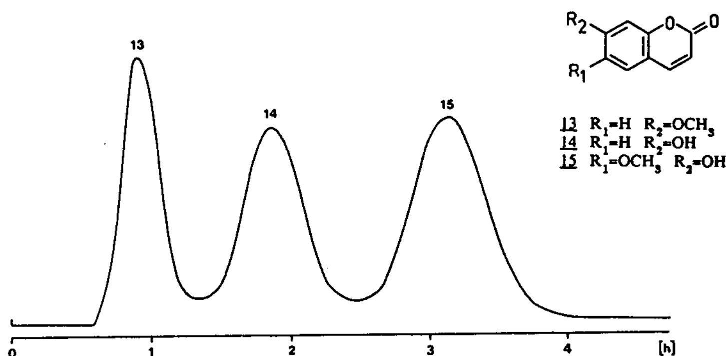

Fig. 6. CPC separation of herniarin (13), umbelliferone (14) and scopoletin (15). Solvent system; light petroleum (b.p. 60–95°C)–ethyl acetate–methanol–water (18:42:30:30); ascending mode; flow-rate, 1.7 ml/min. Rotational speed: 800 rpm. Detection: 254 nm. Sample: 9 mg in 1 ml of upper phase and 0.2 ml of lower phase.

viously used in DCCC[12], gave unsatisfactory results. In contrast, elution with light petroleum (b.p. 60–95°C)–ethyl acetate–methanol–water (18:42:30:30) in the ascending mode resulted in a baseline separation of all three components of the mixture (Fig. 6).

Separation of phenolic acids

Baseline separation of cinnamic (16), ferulic (17) and caffeic (18) acids by CPC was possible with light petroleum–ethyl acetate–methanol–water (18:42:30:30) in the ascending mode (Fig. 7). Thus, the same system was capable of separating both coumarin and phenolic acid mixtures. All three acids were eluted within 2.5 h, with the least polar acid (16) being the first to emerge from the chromatograph and the most polar acid (18) being the last to exit.

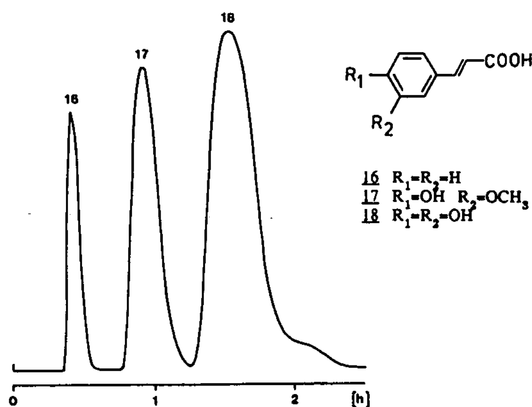

Fig. 7. CPC separation of cinnamic (16), ferulic (17) and caffeic (18) acids. Solvent system as in Fig. 6. Flow-rate: 3.2 ml/min. Rotational speed: 1000 rpm. Detection: 254 nm. Sample: 9 mg in 1 ml of upper phase and 0.2 ml of lower phase.

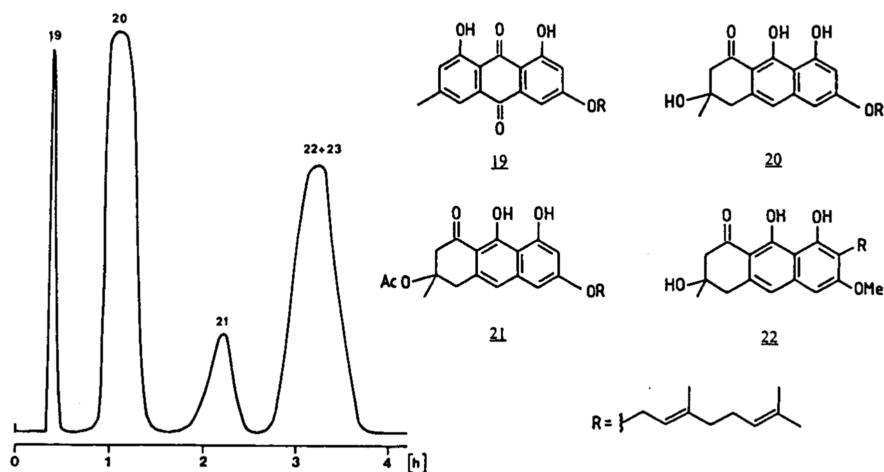

Fig. 8. CPC separation of a light petroleum extract of *Psorospermum febrifugum*. Solvent system: hexane–acetonitrile–methanol (40:25:10); ascending mode; flow-rate, 5.5 ml/min. Rotational speed: 1500 rpm. Detection: 254 nm. Sample: 100 mg in 1 ml of upper phase and 1 ml of lower phase. Ac = Acetyl; Me = methyl.

Separation of anthranoid pigments

The light petroleum extract of the root bark of *Psorospermum febrifugum* (Guttiferae) contains a mixture of anthraquinone, anthrone and tetrahydroanthracene pigments, some of which have strong cytotoxic activities[16]. Separation of these constituents by multi-step flash chromatography and low-pressure reversed-phase

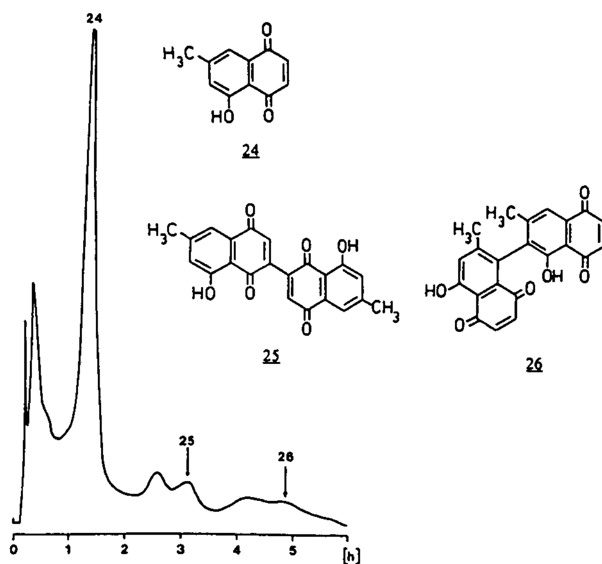

Fig. 9. CPC of a light petroleum extract of *Diospyros usambarensis*. Solvent system as in Fig. 6. Flow-rate: 5.5 ml/min. Rotational speed: 1400 rpm. Detection: 254 nm. Sample: 100 mg in 1 ml of upper phase and 1 ml of lower phase.

liquid chromatography resulted in considerable losses, due to irreversible adsorption on the sorbents. On the other hand, a single CPC step was used to obtain directly three pure compounds (19–21) and a mixture of two anthranoids (22 and a minor component, 23) (Fig. 8), *without* loss of product. With the solvent system hexane–acetonitrile–methanol (40:25:10) in the ascending mode, a 100-mg quantity of the extract was reasonably well separated within 4 h.

Naphthoquinones

A crude light petroleum extract of *Diospyros usambarensis* (Ebenaceae) root bark was injected into the CPC apparatus, and after passage of the organic phase of a light petroleum–ethyl acetate–methanol–water (18:42:30:30) system, 28 mg of pure 7-methyljuglone (24) were obtained (Fig. 9). Furthermore, a partial separation of the naphthoquinone dimers mamegakinone (25) and isodiospyrin (26) was possible.

The liquid–liquid partition one-step isolation method for 7-methyljuglone has certain advantages over chromatographic methods on solid sorbents[16]. Not least of these is the avoidance of losses arising from irreversible adsorption of the naphthoquinone on the packing material. In addition, the elution of compound 24 was complete within 1.5 h.

CONCLUSION

Centrifugal partition chromatography has proved to be a very useful addition to the existing array of liquid–liquid separation techniques. The application of centrifugal force fields to counter-current chromatography drastically shortens the time required for preparative separations of complex mixtures, when compared with DCCC, RLCC, etc. In the absence of a solid sorbent there is no sample loss and, hence, total recovery of injected mixtures. Contamination from solid sorbents is also avoided and there is only minimum decomposition or denaturation of sensitive compounds. Experience with the Sanki CPC apparatus has shown that the resolution is as high as in DCCC and that separations of the order of 100 mg are possible. Mixtures require less than 1 l of mobile phase for total elution and the method is thus inexpensive. Unlike DCCC, droplet formation is not a prerequisite for the choice of solvent systems.

The results presented here demonstrate a variety of separations of natural products, including both polar and non-polar substances: saponins, flavonoids, naphthoquinones, anthranoids and coumarins. Thus, CPC provides a convenient complement to adsorption chromatography. In addition to the features listed, the Sanki CPC instrument is capable of being operated in the reversed-phase mode. This is well illustrated in Fig. 2b by the separation of flavonoids. Changing the elution mode enables a much more rapid elution of hesperetin than would otherwise be possible (Fig. 2a). This leads not only to time saving but also reduces the volume of solvent required.

Experiments are presently underway to extend the range of substances separated by CPC and to investigate the maximum sample loads that may be injected.

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CHROMSYMP. 1408

LARGE-SCALE PURIFICATION OF THE MYCOTOXINS AFLATOXIN B_1, B_2 AND G_1

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SUMMARY

The isolation and purification of gram quantities of the important mycotoxins aflatoxin B_1, B_2 and G_1 are described. The method involves final purification on a Waters Prep LC-500 instrument, loaded with silica cartridges, and elution with chloroform.

INTRODUCTION

The aflatoxins are the most important group of fungal toxins by reason of their frequent contamination of nutrition intended for human and animal consumption, and the extremely severe toxicological effects they exert on many mamalian systems on ingestion[1]. By virtue of this prominence they occupy a pivotal position in mycotoxin research and have been the subject of numerous studies on all aspects of their chemistry.

Chromatographic methodology has traditionally concentrated on the detection and analysis of these metabolites as contaminants of foodstuffs in increasingly smaller amounts, so that many reliable procedures exist for the determination of these compounds at the nanogram level[2]. These methods usually involve chromatography on reversed-phase silica gel which, because of its low capacity, is poorly suited to large-scale separations. However, comparatively little effort has been devoted to investigations at the other end of the scale, in which large quantities of the aflatoxins are produced and purified. Such methods as do exist generally use purification by preparative thin-layer chromatography (TLC) as a final step, because of the poor separation of aflatoxins B_1 (1), B_2 (2), G_1 (3) and G_2 (4) by conventional column chromatography[3]. That procedure is obviously cumbersome for large quantities of material, and the need for aflatoxins B_1, B_2 and G_1 in gram quantities has led us to develop a convenient, rapid and safe method of aflatoxin purification, by chromatography on the Waters Prep LC-500 instrument.

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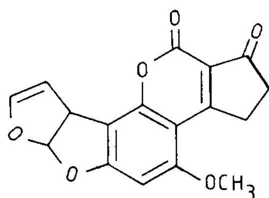

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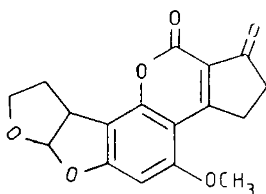

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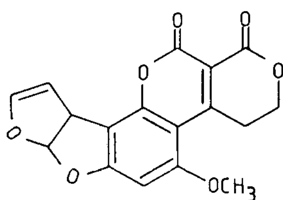

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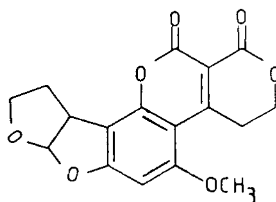

(4)

EXPERIMENTAL

Chemicals and equipment

All solvents used (ethyl acetate, chloroform, methanol, hexane and acetone) were of technical grade, purified by fractional distillation. For gravity column chromatography, Kieselgel 60 (particle size 0.063–0.200 mm) and Aluminiumoxid 90 (particle size 0.063–0.200 mm, Activity II–III) (E. Merck, Darmstadt, F.R.G.) were used. TLC was performed on Merck plates pre-coated with Kieselgel 60 plus a fluorescent indicator to a thickness of 0.25 mm (silica gel 60F-254) developed with chloroform–methanol (97:3, v/v) or chloroform–acetone (9:1, v/v). The conditions used for purification on the Prep LC-500 instrument (Waters, Milford, MA, U.S.A.) were as follows: column, two silica cartridges (250 g); eluent, chloroform; chamber pressure, 35 p.s.i.; solvent pressure, 25 p.s.i.; detector, refractive index; relative response, 20; flow-rate, 100 ml/min; chart speed, 5 min/cm.

Aflatoxin production

Aspergillus flavus (CSIR 840) was grown at 27°C for 5 days in stationary culture in the M_1 medium (47 l) consisting of sucrose (200 g), yeast extract (20 g), $MgSO_4 \cdot 7H_2O$ (10 g), $ZnSO_4 \cdot 7H_2O$ (26 mg), $CuSO_4 \cdot 5H_2O$ (2.6 mg), $Co(NO_3)_2 \cdot 6H_2O$ (1.3 mg) and $FeSO_4$ (5 mg) (per litre) in 500-ml flasks each containing 100 ml of medium. In preliminary growth experiments, this strain had been found to produce high levels of aflatoxins B_1 and G_1 and relatively smaller quantities of aflatoxin B_2, and practically no aflatoxin G_2. Ethyl acetate (100 ml) was added to each flask of the fungal broth in order to kill spores before filtration. The resultant fractions were treated separately.

Preliminary work-up

Media fraction. This fraction was separated into two phases. The aqueous phase was extracted with ethyl acetate (2 × 5 l) and the organic phases were pooled. The aqueous phase was adjusted with sodium hydroxide pellets to pH 10, to destroy unextracted aflatoxins, and then discarded. The organic phase was dried (sodium sulphate), filtered and evaporated. The residue was partitioned between chloroform and water (4 l, 1:1, v/v) and the aqueous phase was again treated with sodium hydroxide before it was discarded. The chloroform phase was filtered to remove crystalline material (Kojic acid), dried (sodium sulphate), filtered and evaporated to give a solid residue (21.0 g) hereafter referred to as fraction A.

Mycelial fraction. The mycelial material was homogenized for 5 min in a Waring blender with chloroform-methanol (1 l, 1:1, v/v). The resultant slurry was filtered, and the mycelial fragments were again homogenized using the same conditions. After filtration, the filtrates were pooled and evaporated and the mycelial remains were treated with 1 *M* sodium hydroxide (1 l) before they were discarded. The residue was partitioned between 90% methanol and *n*-hexane (1 l, 1:1, v/v), and the 90% methanol layer was evaporated. The residue was partitioned between chloroform and water (1 l, 1:1, v/v); the chloroform layer was dried (sodium sulphate), filtered and evaporated to give a solid residue (9.7 g) hereafter referred to as fraction B. The *n*-hexane and water fractions were treated with sodium hydroxide before they were discarded.

Preliminary clean-up

Fractions A and B were pooled and purified on a silica gel gravity-fed column (10 cm I.D.) (1.7 kg silica) and eluted with chloroform–acetone (10 l) (95:5, v/v). The eluates, hereafter referred to as fractions C (11.3 g, containing aflatoxins B_1 and B_2, as well as several unidentified pigments) and D (3.2 g, containing aflatoxins B_1, B_2 and G_1 but no pigments) were collected and evaporated to dryness. Fraction C was further purified by chromatography on alumina (600 g), eluting with chloroform–acetone (80:20, v/v) to give a Fraction E (1.18 g, containing only aflatoxins B_1 and B_2) as a cream coloured powder.

Purification on Waters Prep LC 500

Fraction E. The entire fraction in chloroform (5 ml) was purified by a single passage through the system at a flow-rate of 100 ml/min. When the refractive index detector indicated the beginning of the aflatoxins peak (about 8 min), 50-ml fractions were collected and monitored by TLC[4]. In this way, pure aflatoxin B_1 (700 mg), pure aflatoxin B_2 (80 mg) and a mixture of aflatoxins B_1 and B_2 (320 mg) were obtained. The mixture was hydrogenated to give pure aflatoxin B_2 (280 mg)[5].

Fraction D. The entire fraction in chloroform (5 ml) was purified by a single passage through the Waters system at a flow-rate of 100 ml/min. The chromatographic trace is shown in Fig. 1. Collection of 50-ml fractions, as above, monitored by TLC, gave pure aflatoxin B_1 (390 mg), pure aflatoxin G_1 (2.1 g), a mixture of aflatoxins B_1, B_2 and G_1 (150 mg) and a mixture of aflatoxin B_1 and an impurity of lower R_F (450 mg). Total yields of aflatoxins: B_1 (1.09 g), B_2 (360 mg), G_1 (2.1 g). The purity of the aflatoxins was checked by elemental analysis, and by ^1H and ^{13}C NMR spectroscopy at 500 and 125 MHz, respectively.

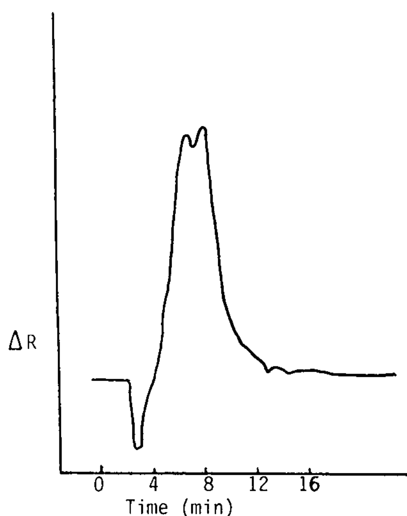

Fig. 1. Purification of fraction D on the Waters Prep 500. ΔR is the difference in refractive index between the column effluent and the eluting solvent.

Precautions

Standard precautions necessary when working with aflatoxins were observed. These included the mandatory use of gloves and protective clothing, the performance of all work involving fungal extractions in a fume-hood and the washing of all used glassware and benchtops with sodium hypochlorite solution.

DISCUSSION

The above methodology provides easy access to large quantities of aflatoxins B_1, B_2 and G_1. The method is rapid and convenient, and obviates the use of preparative TLC plates, which becomes cumbersome with larger amounts of material. Moreover, purification with the Prep LC-500 system also removes the need for scraping off bands from the preparative plates and the concomitant risk of breathing in silica impregnated with aflatoxins. With the recycle facility available on the machine, the method can easily be scaled up for even larger amounts of sample.

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CHROMSYMP. 1409

Note

Preparative liquid chromatographic separation of isomers of prostacyclin carba-analogues and their intermediates

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The carba-analogues of prostacyclin are its chemically stable and biologically active analogues[1] and a significant number of these compounds have been synthesized and tested for biological activity[2]. It is clear that every variation in their configuration causes a drastic change in their activity[3,4]. Moreover, in many of the synthetic stages the stereochemistry of these analogues is not completely controllable by chemical methods[5]. Therefore, problems arise in of the preparative separation of the mixtures of isomers formed.

In the synthesis of the carba-analogues of prostacyclin mainly three types of isomers can be distinguished, requiring chromatographic separation[5,6]: (1) regio isomers formed in the epoxide ring-opening reactions with various organometallic reagents; (2) *E*/*Z* geometric isomers formed by the Wittig olefination of ketones; and (3) α/β-stereoisomers at the C-15 position[5,6] of the prostacyclin molecule (in this instance chemical control over the isomer ratio is completely lacking).

Most of these isomers can be successfully resolved, but the separation of the C-15 α/β-stereoisomers of the 13,14-didehydro carba-analogues requires the formation of cobalt complexes prior to column chromatography (this lengthens the synthetic process)[7,8].

Among the factors determining the resolution and, consequently, the loadability, throughput and cost in preparative separations, the selectivity of resolution (separation factor, α) is of primary importance[9,10]. Literature data on the selectivity of resolution of the above isomers are not available (mostly silica gel was used as the stationary phase, but different mobile phases have been used by different investigators). Therefore, we have studied the selectivity of resolution of these isomers. We also show that this approach has been successful in the semi-preparative separation of the C-15 α/β-isomers of a new 13,14-didehydro carba-analogue of prostacyclin without the use of cobalt complexes.

EXPERIMENTAL

All the compounds studied were synthesized at the Institute of Chemistry of the Academy of Sciences of the Estonian S.S.R. (see Fig. 1). Their chemical identities were

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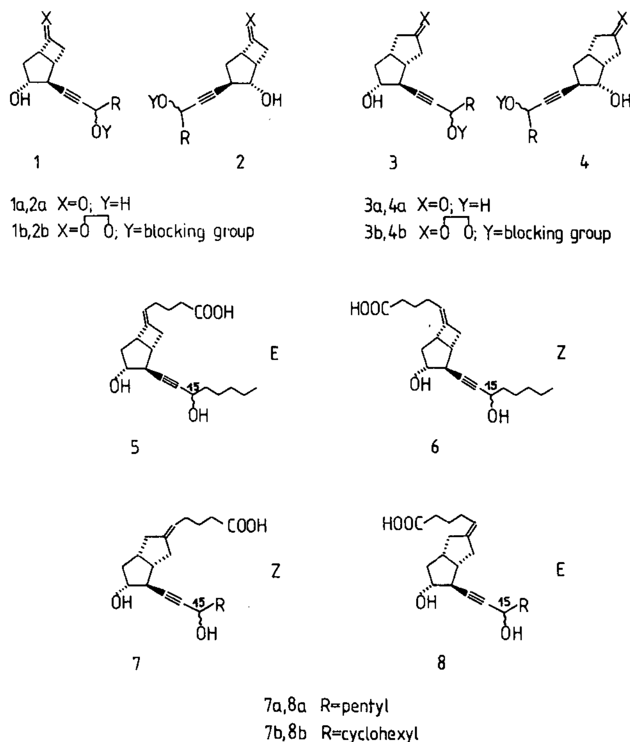

Fig. 1. Formulae of the compounds studied.

established by ^{13}C NMR spectroscopy. Separations were performed on a DuPont Model 8845 liquid chromatograph, equipped with UV spectrophotometric and refractometric detectors.

Separation factor measurements were performed with a Zorbax SIL (DuPont) analytical column (150 mm × 4.6 mm I.D.). The semi-preparative resolution of compounds 7b and 8b was performed on two Zorbax SIL Golden Series columns (80 mm × 6.2 mm I.D.) coupled in series. Their total plate number was 12 500 at a flow-rate of 1.2 ml/min, as calculated by using the naphthalene peak.

All the solvents used were purchased from Reakhim (Moscow, U.S.S.R.). Their preparation before use is described in ref. 11.

RESULTS AND DISCUSSION

The resolution of the regio isomers formed is usually performed at the stage of blocked (1a–4a) or unblocked (1b–4b) ketonediols. The selectivity of their resolution on silica gel has been reported[12]. It has been shown that the separation factor, α, varies significantly depending on the localization parameters m and m^0 and on the hydrogen bonding between solutes and mobile phases. It is essential to note that a selectivity of resolution of up to $\alpha = 2.0$ can be achieved. This means that only 100 theoretical plates

are necessary for their complete resolution, and sample loads of up to 10 mg per gram of adsorbent (50–100 μm) can be applied[10].

The selectivity of resolution of the *E*/*Z* isomers of prostacyclin carba-analogues has been reported previously[13]. It has been shown that the selectivity depends greatly on the structure of the isomers. In one instance it exceeded 2.0 on silica gel, but often low α values are encountered[13]. Therefore, we examined the solvent selectivity on the example of compounds 5 and 6 (the most difficult to resolve pair of *E*/*Z* isomers) using binary mobile phases A–B (modified with water at concentrations up to saturation in order to avoid peak tailing and loss of efficiency of resolution[11]), where A is, *n*-hexane, benzene or chloroform and B is 2-propanol, ethanol, methanol, acetonitrile, acetone or ethyl acetate. The α value in the solvent systems studied varied from 1.08 to 1.19, thus increasing the loadability significantly and resulting in savings on scaling up the resolution (data for some mobile phases yielding higher selectivities of resolution are presented in Table I).

TABLE I

CAPACITY FACTORS (k') AND SELECTIVITY OF RESOLUTION (α) OF COMPOUNDS 5 AND 6

Column, Zorbax SIL (150 mm × 4.6 mm I.D.); temperature, 35°C; flow-rate, 0.6 ml/min.

Mobile phase	k'		α
	Compound 5	*Compound 6*	
Benzene–2-propanol–water (97:2.95:0.05)	8.30	9.88	1.19
Chloroform–2-propanol–water:			
95:4.94:0.06	4.20	4.90	1.18
96:3.94:0.06	7.43	8.56	1.18
Chloroform–ethanol–water:			
96:3.83:0.17	4.32	4.94	1.15
97:2.87:0.13	8.98	10.5	1.18
n-Hexane–2-propanol–water			
96:3.86:0.14	5.37; 5.50	6.03; 6.22	1.10
97:2.9:0.1	18.3; 18.6	21.4; 22.0	1.14

However, as shown in Table I, only with *n*-hexane–2-propanol as the mobile phase can resolution of the C-15 α/β-isomers of 13,14-didehydro analogues be achieved. The four-membered ring carba-analogues 5 and 6 were not identified because the resolution is still insufficient for their preparative resolution. However, as shown previously[13], the five-membered ring carba-analogues 7a and 8a can be resolved with a resolution of $R_s = 1.0$ on a column having 9700 theoretical plates. In this study, we resolved semi-preparatively the C-15 α/β-isomers of carba-analogues 7b and 8b (Fig. 2). Thus, 24 mg of crude synthetic mixture (previously filtered through silica gel) afforded, on eight injections, 2–4 mg of each of the four isomers of the new prostacyclin carba-analogues 7b and 8b in pure form and allowed the determination of their biological activities. It should be noted that this is a case where the resolution of *E*- and *Z*-isomers also requires the use of high-performance liquid chromatography

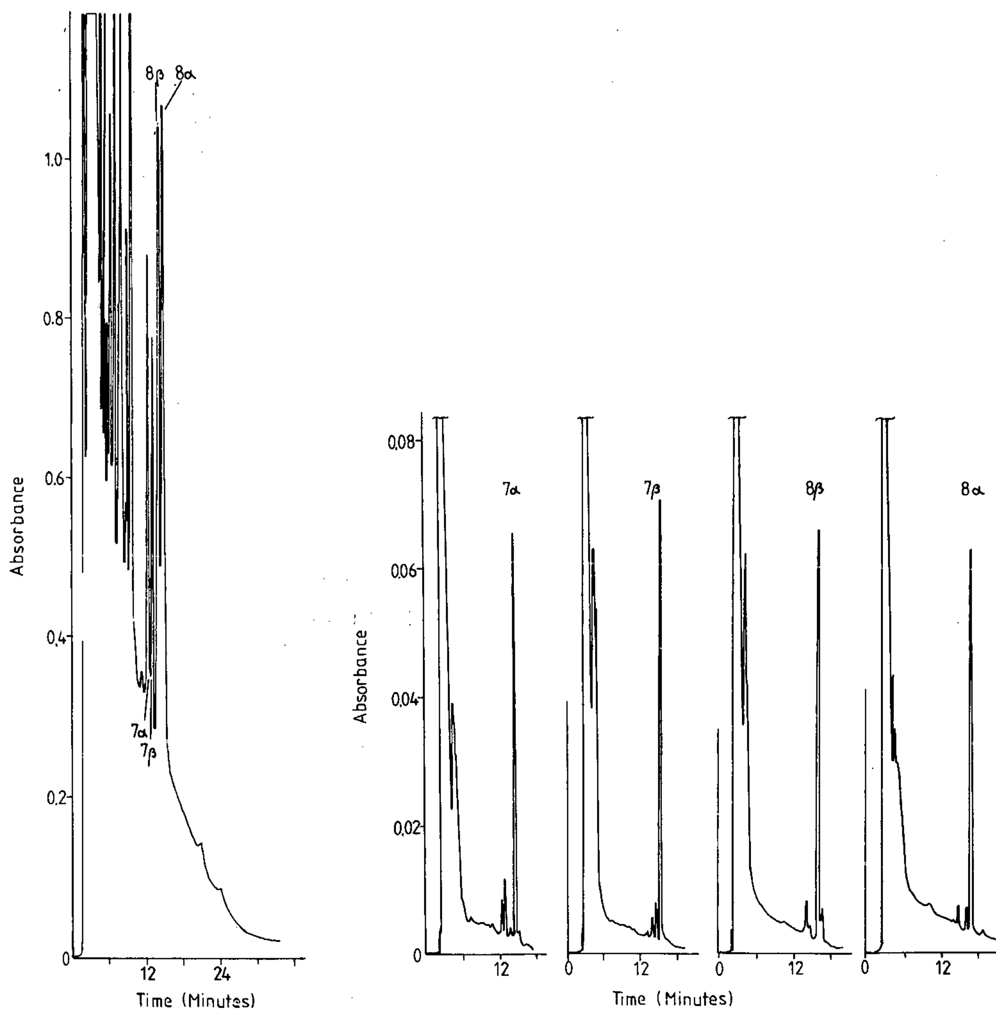

Fig. 2. Semi-preparative resolution of the C-15 α/β-isomers of compounds 7b and 8b. Columns, two Zorbax SIL Golden Series columns (80 mm × 6.2 mm I.D.) in series; mobile phase, *n*-hexane–2-propanol–water (90:9.9:0.1); flow-rate, 1.2 ml/min; detection, 210 nm; sample size, 3 mg injected in 50 μl of benzene. α and β refer to the corresponding C-15 isomers.

Fig. 3. Purity of the C-15 α/β-isomers of compounds 7b and 8b. Sample size, 25 μg injected in 5 μl of benzene. Other chromatographic conditions as in Fig. 2.

(HPLC). The isomers were checked for purity on the same columns under analytical conditions (Fig. 3). It is apparent that the purity of throughput could easily be increased by using a column (or columns of nearly equal performance connected in series) having up to 20 000 theoretical plates. For this purpose, a column (or columns) packed with 5-μm particles may be used.

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CHROMSYMP. 1402

Note

Isolation of tocopherol homologues by preparative high-performance liquid chromatography

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Natural vitamin E from vegetable oils is comprised of a mixture of D-α-, β-, γ- and δ-tocopherols (Fig. 1). We were especially interested in isolating considerable quantities of D-γ-tocopherol with a purity of >90%. Preparative high-performance liquid chromatography (HPLC) seemed to be most appropriate for this objective. HPLC is a convenient method for tocopherol analyses[1–4]. Unmodified silica is usually used as the stationary phase and hexane or octane with a polar modifier as the eluent[3].

EXPERIMENTAL

For the isolation of natural D-γ-tocopherol we used a 400 mm × 100 mm column of 25–40 μm silica and the Prepbar 100 system of Merck (Darmstadt, F.R.G.). The fractions collected were evaporated in a 20-l rotary evaporator. The experimental conditions are summarized in Table I.

RESULTS

Our starting material was a vegetable oil extract from plant seeds with a total tocopherol content of 70%, 60% of which was D-γ-tocopherol (Fig. 2). A direct scale-up from the analytical to the preparative mode was achieved by only slight

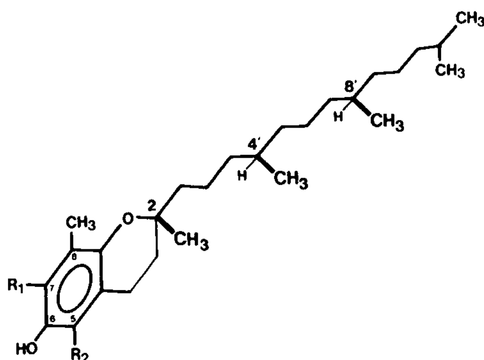

Fig. 1. Structures of the natural tocopherols: α, R_1, R_2 = CH_3; β, R_1 = H, R_2 = CH_3; γ, R_1 = CH_3, R_2 = H; δ, R_1, R_2 = H.

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TABLE I

EXPERIMENTAL CONDITIONS FOR ANALYTICAL AND PREPARATIVE HPLC

	Analytical HPLC	*Preparative HPLC*
System/pump	Kontron 420	Merck Prepbar 100
Column	250 mm × 4 mm	400 mm × 100 mm
Stationary phase	LiChrosorb Si 60, 7 μm	LiChroprep Si 60, 25–40 μm
Eluent* (*n*-hexane–*tert.*-butyl methyl ether)	96:4 (v/v)	97:3 (v/v)
Flow-rate (ml/min)	2	450
Injection volume	15 μl	50 ml
Sample amount**	1 μg	15 g
Detection	220 nm	205 nm, split 1:4
Total analysis time (min)	40	60

* HPLC solvents were used (Promochem, Wesel, F.R.G.).
** Sample dissolved in the HPLC eluent.

changes in the chromatographic conditions (Table I). Sometimes, prepurification of the crude oil extract by filtration through silica with hexane as the solvent may be helpful to prevent fouling of the preparative column. However, in our case, this was not necessary.

Whereas the efficiency of the analytical column was sufficient for separating the D-β and D-γ isomers (Fig. 2), they seemed to be overlapped in the preparative chromatogram (Fig. 3). This was due to overloading the column with 15 g of the tocopherol starting mixture in an injection volume of 50 ml. Nevertheless, suitable fractionation (Fig. 3) allowed the isolation of up to 4 g of natural D-γ-tocopherol per

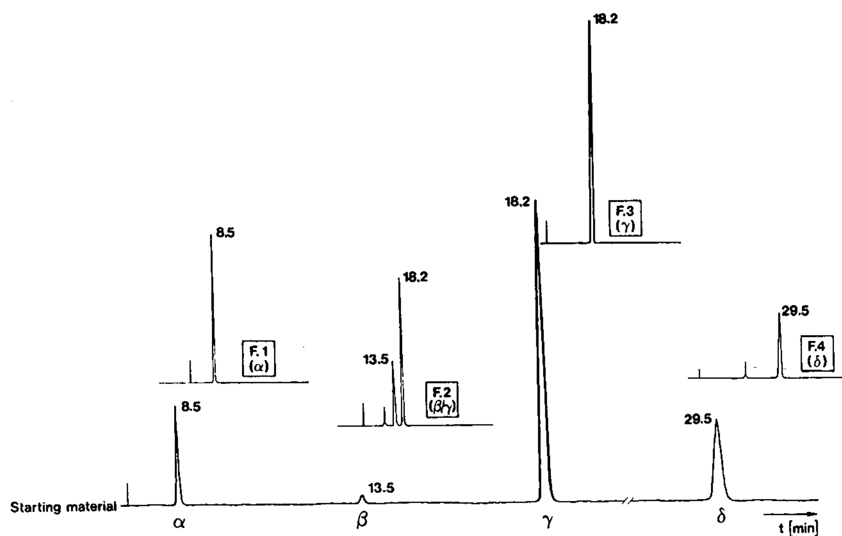

Fig. 2. Analytical HPLC of the starting material and of the D-α (F.1) to D-δ (F.4) fractions, isolated by preparative HPLC.

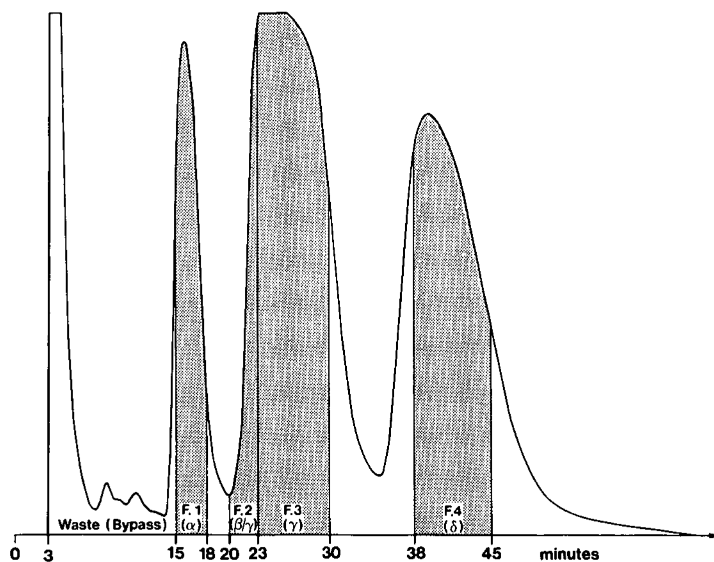

Fig. 3. Preparative chromatogram with time marks for the cuts of fractions F.1–F.4.

TABLE II

ANALYTICAL DATA FOR ISOLATED NATURAL D-TOCOPHEROL FRACTIONS FROM 350 g STARTING MATERIAL CONTAINING 70% TOTAL TOCOPHEROL, 60% OF WHICH WAS D-γ-TOCOPHEROL

Fraction	*Content* (%, w/w)*				*Yield (g)*
	D-α	D-β	D-γ	D-δ	
(1) D-α	88.2	–	–	–	20
(2) D-β	2.5	10.4	39.0	–	8
(3) D-γ	–	–	95.4	–	95**
(4) D-δ	–	–	3.6	90.1	49

* HPLC data, calibrated with tocopherol standards from Merck.

** 65% D-γ-Tocopherol yield relative to the D-γ-tocopherol content of the starting material.

experiment in 95.4% purity, without contamination by other tocopherols. The analytical data for the fractions isolated are summarized in Table II.

Thus, preparative HPLC has proved to be a rapid and economic method for isolating natural tocopherol homologues.

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CHROMSYMP. 1412

ISOLATION OF GENTAMICIN C COMPOUNDS FROM CULTURE FILTRATES OF *MICROMONOSPORA PURPUREA*

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SUMMARY

A liquid chromatographic method was developed for the isolation of gentamicin C compounds from commercial fermentation products in order to monitor health hazards (oto- and nephrotoxicity). Chromatography was carried out on silica gel 60 (15–40 μm) with a medium-pressure chromatographic system, employing methanol–25% ammonia solution (85:15, v/v) and methanol–chloroform–25% ammonia solution (20:10:5, v/v) as mobile phases. The eluted fractions were neutralized with 1.0 *M* hydrochloric acid, concentrated *in vacuo* and desalted by gel filtration. It was possible to demonstrate by ^{1}H NMR spectroscopy and high-performance liquid and thin-layer chromatography that the separated fractions contained components C_1, C_{1a} and C_2 in purities of more than 95%.

INTRODUCTION

Gentamicin is a broad-spectrum, basic, water-soluble aminoglycoside antibiotic mixture produced by *Micromonospora purpurea*[1–4]. The isolation of the gentamicins from fermentation broth can be performed by several methods[5–15] and, depending on the procedure, it is possible to extract the whole antibiotic complex or some of its components. Thirteen gentamicin compounds have been isolated and identified. The most important method is extraction by ion-exchange chromatography on cation- and anion-exchange resins, such as Amberlite IRC-50, CG-50, CG-120 and Dowex 1X2. It is possible to isolate the three major components C_1, C_{1a} and C_2 on cellulose powder[6,7], whereas the other C compounds (C_{2a} and C_{2b}) can be extracted by Craig counter-current distribution and represent only 4% of the total C complex[11,12]. Column chromatography on silica gel has been employed for the isolation of the major gentamicin components A, B, B_1 and X[8]. The separation of the gentamicin C compounds on silica gel was not satisfactory[15].

The aim of this investigation was to develop a medium-pressure liquid chromatographic (MPLC) method on silica gel for the isolation of pure major gentamicin components.

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EXPERIMENTAL

Materials and reagents

Gentamicin samples were obtained from Merck (Darmstadt, F.R.G.) and Serva (Heidelberg, F.R.G.). Purified components were made available as standards by G. H. Miller (Schering , Bloomfield, NJ, U.S.A.). The USP gentamicin standard (microbiological activity, 663 μg/mg; C_1, 36.9%; C_2, 31.6%; and C_{1a}, 31.5%) was obtained from United States Pharmacopeial Convention (Rockville, MD, U.S.A.). All other chemicals employed were supplied by Merck or Baker (Gross-Gerau, F.R.G.).

MPLC equipment

The chromatographic equipment consisted of a Büchi B-681 pump (Büchi, Göppingen, F.R.G.) with an injection valve (2-ml loop) and a Büchi B-684 fraction collector. Columns (23 × 2.6 cm I.D.) were packed in the laboratory with silica gel 60 (15–40 μm) for column chromatography (Merck).

Mobile phases

Mobile phase A consisted of methanol–25% ammonia solution (85:15, v/v) and mobile phase B consisted of methanol–chloroform–25% ammonia solution (20:10:5, v/v/v), both pumped through the column at flow-rate of 25 ml/min.

Detector system

The detection of the gentamicins was carried out by high-performance thin-layer chromatography (HPTLC) and derivatization with ninhydrin. A volume of 6 μl of each eluted fraction was applied with microcaps (Drummond, Broomall, U.S.A.) to an HPTLC silica gel plate (10 × 20 cm, Merck). After one-dimensional development with a solvent system consisting of chloroform–methanol–32% ammonia solution (10:8:5, v/v/v), the spots were detected by ninhydrin derivatization by dipping the well dried plate into a 0.2% (w/v) methanolic ninhydrin solution and heating for 10 min at 110°C.

MPLC procedure

Gentamicin sulphate (1 g) was dissolved in 6 ml of water–methanol (5:1; v/v) and a 2.0-ml volume of this solution was injected into the chromatographic system. After passage of 200 ml of mobile phase, the effluent was collected in fractions of 20 ml.

Gel filtration

The eluted fractions were neutralized with 1.0 *M* hydrochloric acid and concentrated in *vacuo*; 200-mg samples of the residues obtained were dissolved in 2 ml of water and purified by gel chromatography on Bio-Gel P-2 (450 × 22 mm) (Bio-Rad Labs., Munich, F.R.G.).

^1H NMR spectrometry

The spectrometry investigations were carried out in deuterium oxide with sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 as internal reference (Bruker AM 400 Multinucleus Fourier NMR apparatus, 400 MHz) (Bruker, Karlsruhe, F.R.G.).

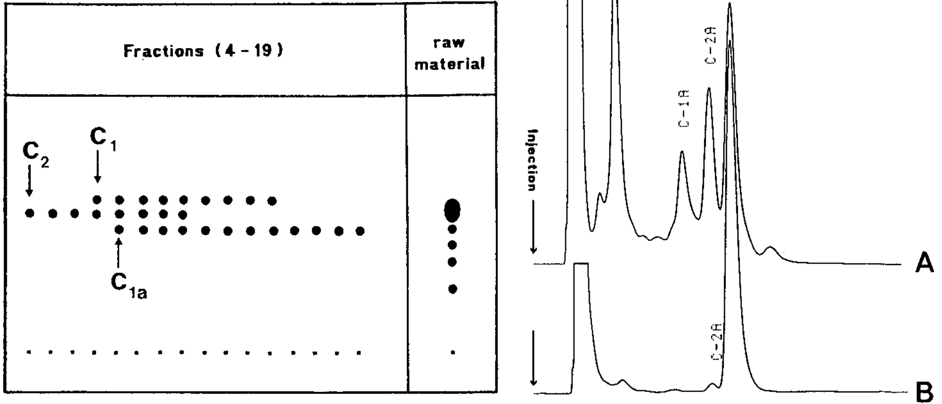

Fig. 1. Elution of the gentamicin components with mobile phase A in comparison with those of the raw material and pure components.

Fig. 2. HPLC of (A) the gentamicin C complex and (B) the eluted C_2 fraction according to Weigand and Coombes[16].

RESULTS AND DISCUSSION

With mobile phase A C_2 was eluted first and could be isolated from fractions 4–6 (Fig. 1). Fractions 7–11 were collected, neutralized, concentrated *in vacuo* and chromatographed again under the same conditions. Mobile phase B was then employed to isolate C_1 and C_{1a} from the concentrated extract of fractions 12–19.

Fig. 3. HPTLC of (A) the gentamicin C complex and (B) the eluted C_2 fraction according to Kunz and Jork[35].

Under these conditions, the elution sequence changed ($C_1 \rightarrow C_2 \rightarrow C_{1a}$) and C_1 could be completely separated from C_{1a}.

The purity of the separated components was monitored by high-performance liquid chromatography (HPLC), HPTLC and ^1H NMR spectroscopy. Fig. 2 illustrates the HPLC separation of the gentamicin C complex (A) and the eluted C_2 fraction (B), carried out by the method of Weigand and Coombes[16]. This HPLC procedure was the most satisfactory[17–35]. C_1 and C_{1a} were of a comparable degree of purity (results not shown). Analogous results were obtained by HPTLC (see Figure 3)[35]. ^1H NMR spectroscopy showed no evidence of any contamination of the isolated C compounds.

In conclusion, it has been possible to isolate the three major components of the gentamicin complex in high purity with one chromatographic system by combining two mobile phase systems.

ACKNOWLEDGEMENTS

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CHROMSYMP. 1403

RAPID AND EFFICIENT METHOD FOR THE ISOLATION AND CHARACTERIZATION OF PLANT AROMATIC CHOLINE ESTERASES

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SUMMARY

Aromatic choline esterases have been isolated from seedlings of six crucifers. A fast polymer/protein liquid chromatographic (FPLC) method was developed for the final purification and characterization of the enzymes. FPLC anion-exchange chromatography and chromatofocusing resulted in highly purified enzymes. The elution profiles of the enzymes from different plant species showed appreciable differences. Chromatofocusing revealed differences in their relative p*I* values (4.3–5.1) and allowed the separation of aromatic choline esterase isoenzymes. Chromatofocusing revealed that two isoenzymes of aromatic choline esterases occur in *Barbarea intermedia*. The molecular weight of the enzyme from *Sinapis alba* was calculated to be about 40 000 daltons by gel filtration. Kinetic studies confirmed the existence of different aromatic choline esterases in plants.

INTRODUCTION

Aromatic choline esters are a group of natural products widespread in glucosinolate-containing plants (*e.g.*, crucifers)[1–7] and orchidaceae[8]. Often they accumulate appreciably in the seeds, sinapine being the best known example in *Brassica*[9]. Several other types of aromatic choline esters can be quantitatively dominant among the aromatic choline esters[9]. Examples of dominating aromatic choline esters in other plants are *p*-hydroxybenzoylcholine in *Sinapis*[7], sinapine glucoside in *Alliaria*[5], isoferuloylcholine[3] in *Barbarea*[10] and a lignan type in *Brassica nigra*[10].

During germination of the seeds, the aromatic choline esters are catabolized[7,11]. The initial step is the hydrolysis of the esters, which is catalysed by aromatic choline esterases[11]. Knowledge about the enzymes is sparse and based almost solely on sinapine esterase isolated from seedlings of *Brassica hirta* Moench.[12] and *Raphanus sativus* L.[13]. Additional information about aromatic choline esterases is therefore needed, especially owing to the increasing interest in aromatic choline esters and products thereof in relation to the quality of oilseed rape[9,11].

In this work, aromatic choline esterases were isolated from the seedlings of six crucifers. The properties of highly purified enzymes were investigated and compared

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with previously reported data for sinapine esterase. A combination of fast polymer/protein liquid chromatographic (FPLC) techniques has been developed for the purpose of obtaining rapid information about the status of aromatic choline esterases in various crops. The application of FPLC resulted in a rapid and effective method for the study of the presence and properties of these enzymes, including the separation of isoenzymes. Further, a comparison of the kinetic parameters for the enzymes, including five different substrates found in varying amounts in the seeds of the six plants, was performed for a more detailed characterization of the differences between the enzymes.

EXPERIMENTAL

Plant material

Seedlings were produced as described elsewhere[11] from *Sinapis alba* L., *Brassica napus* L. cv. Line and cv. Gulliver, *Brassica nigra* (L.) Koch cv. Junius, *Brassica campestris* L. cv. Candle and *Barbarea intermedia* Bor.

Determination of aromatic choline esters

HPLC methods described elsewhere were used[9,14].

Enzyme assay

Sinapine thiocyanate (100 μM) was prepared in McIlvaine buffer (pH 6.50); the total volume was 1545 μl. After addition of enzyme, dA_{350}/dt was measured against buffer. One enzyme unit (U) = 1 μmol of sinapine hydrolysed per minute (25°C), resulting in

$$\text{U/ml enzyme solution} = \frac{1545}{b\,(\varepsilon_{350,\text{sinapine}} - \varepsilon_{350,\text{sinapic acid}})} \cdot \frac{-dA}{dt} = \frac{1.545}{8b} \cdot \frac{-dA}{dt}$$

where cell path length = 1.00 cm, $\varepsilon_{350,\text{sinapine}} = 8900\ \text{l mol}^{-1}\ \text{cm}^{-1}$, $\varepsilon_{350,\text{sinapic acid}} = 900\ \text{l mol}^{-1}\ \text{cm}^{-1}$ and b = ml of enzyme solution used in assay.

Protein determination

The Coomassie Brilliant Blue method with bovine serum albumin as a standard was used[15]. Fractions from FPLC were measured at 280 nm.

Enzyme spot test

Acetylthiocholine iodide in McIlvaine buffer (pH 6.50) (75 mM, 5 μl), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent; Sigma, St. Louis, MO, U.S.A.) in McIlvaine buffer (pH 8.00) (10 mM, 25 μl) and a maximum of 100 μl of enzyme solution were mixed with McIlvaine buffer (pH 8.00) in a total volume of 230 μl[11]. The colours were compared visually after 20 min.

Crude extract

Lyophilized seedlings (10 g) were homogenized in deionized water (150 ml; 4°C) by use of an Ultra Turrax homogenizer (5 × 20 s). Centrifugation for 60 min at 11 000 g (4°C) and filtration through filter-paper yielded about 120 ml of crude extract.

Ammonium sulphate precipitation

Addition of solid ammonium sulphate and magnetic stirring at 4°C for 30 min, followed by centrifugation for 60 min at 11 000 *g* (4°C), yielded a precipitate between 21 and 63% of salt, which was dissolved in 30 ml water and centrifuged for 2 h as above. The supernatant contained the aromatic choline esterases.

Ultrafiltration

For concentration and removal of salt, the supernatant from the ammonium sulphate purification step was diluted to 100 ml with water, followed by reduction of the volume to 30 ml in a PM 30 ultrafiltration unit (Amicon, Danvers, MA, U.S.A.). Water (70 ml) was added and the volume was reduced to 10 ml. The filter was washed with 10 ml of 0.1 *M* sodium chloride solution, followed by 10 ml of water. The wash solutions were added to the 10 ml of concentrated solution and the mixture was lyophilized.

Gel chromatography

The powder from the ultrafiltration was dissolved in 10 ml of water (magnetic stirring for 30 min) and the solution was centrifuged for 1 h at 20 000 *g* (4°C). The supernatant (4–5 ml) was applied to a gel chromatographic column (Sephadex G-75; 100 × 2.6 cm I.D.) (Pharmacia, Uppsala, Sweden), equilibrated with McIlvaine buffer (pH 6.50). This buffer was used for elution (10-ml fractions; flow-rate 60 ml/h). The fractions containing aromatic choline esterase activity were pooled, and the salt content was reduced to 1/2000 by means of the ultrafiltration unit (see above) and addition of water.

Isolation of aromatic choline esterase by FPLC

All instrumentation was obtained from Pharmacia, and consisted of a gradient programmer (GP-250), a chromatography rack, a single-path monitor (UV-1 at 280 nm), a fraction collector (FRAC-100), a two-channel recorder (REC 482), two high-precision pumps (P-500) and a valve (V-7).

FPLC anion-exchange chromatography. A prepacked column (Mono Q HR 5/5, 5 cm × 5 mm I.D.; Pharmacia) equilibrated with buffer A (20 m*M* Tris, pH 7.60) was used. Buffer B was 20 m*M* Tris–1.0 *M* sodium chloride (pH 7.60). Gradient elution was performed with 2 ml of A; 10 to 35% B in 20 min; 2 ml of B; 2 ml of A; 1 ml/min; 1 ml per fraction. To perform ion-exchange chromatography on the G-75 preparation, the freeze-dried product was dissolved in 4.0–6.0 ml of buffer A and the solution was filtered through a 0.4-μm filter (Millipore, Milford, MA, U.S.A.). Two or three consecutive chromatograms of 1.0-ml samples were obtained.

FPLC chromatofocusing. A prepacked Mono P HR 5/20 column (20 cm × 5 mm I.D.; Pharmacia) was equilibrated with buffer A [25 m*M* bis-Tris, pH 6.30 (adjusted with hydrochloric acid)]. The column was eluted with 3 ml of buffer A followed by 46 ml of buffer B. [Polybuffer 74 (Pharmacia) diluted with water (1:9) (pH 3.70) (adjusted with hydrochloric acid)]. The flow-rate and fraction size were as above. A 200–500-μl volume of the purest fractions from the ion-exchange chromatogram were applied to the column after filtration through a 0.4-μm filter. The pH of the fractions was measured with a glass electrode (Radiometer, Copenhagen, Denmark). After each run, the column was rinsed with 1.0 ml of 2 *M* sodium chloride solution.

Molecular weight

Gel chromatography (Sephadex G-200; Pharmacia) was performed on two columns (30 × 1.5 cm I.D.) combined in series. McIlvaine buffer (pH 6.50) was used to dissolve the sample and for elution (5 ml/h). The reference compounds used were blue dextran (2 × 10^6 daltons; Pharmacia), and gel filtration standard (Bio-Rad Labs., Richmond, CA, U.S.A.) containing five compounds with molecular weights from 670 000 to 1350 daltons.

Optimum pH

Assay mixtures were as mentioned above, except for the McIlvaine buffers (pH 3.00, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50 and 8.00). At 5-min intervals, 150-μl samples were mixed with 150 μl of 2 *M* perchloric acid and, after centrifugation, 90-μl portions of the supernatant were used for sinapine determination by isocratic HPLC[9]. A correction for non-enzymatic hydrolysis of sinapine at alkaline pH was found not to be necessary.

Gel electrophoresis

Polyacrylamide gel (7%; Tris buffer, pH 8.40) (Bio-Rad Labs.) was used. After electrophoresis, some gels were stained for proteins with Coomassie Brilliant Blue. Other gels were placed for 16 h in a developing solution [70 m*M* maleic acid–170 m*M* sodium sulphate–4 m*M* copper(II)sulphate pentahydrate–20 m*M* glycine–3 m*M* magnesium chloride–4 m*M* acetylthiocholine iodide; pH 6.50]. By this method, which is a modification of the procedure of Brock and Hayward[16], bands containing choline esterase activity became visible as a white precipitate of copper thiocholine.

Kinetics

McIlvaine buffer (pH 6.50) was used throughout the studies. The substrates used are given in Table I. The activity was determined for at least five concentrations in the following ranges: sinapine, 5–105 μ*M*; isoferuloylcholine and *p*-coumaroylcholine, 3–15 μ*M*; and hesperalin and *p*-hydroxybenzoylcholine, 3–25 μ*M*.

Hydrolysis was monitored in a thermostated (25°C) MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan) by recording dA/dt at a fixed wavelength (see below).

Substrate solution (1500 μl in buffer) was added to a fixed amount of enzyme (30–100 μl) and dA/dt was followed for about 6 min, except for *p*-hydroxybenzoylcholine (40 min), at the wavelengths shown in Table I. v_0 (μmole/min) was obtained from the equation

$$v_0 = (1500 + b)\,(-dA/dt)(1/\varepsilon)$$

where b = μl of enzyme added, v_0 = initial velocity, t = time (min), $\varepsilon = \varepsilon_{\text{choline ester}} \cdot \varepsilon_{\text{acid}}$ and cell path length = 1 cm.

The purified enzymes from the FPLC ion-exchange chromatogram were used for kinetic studies; for *B. napus* cv. Line, the ultrafiltered ammonium sulphate precipitate was used. The crude extract from *S. alba* was used for hydrolysis of *p*-hydroxybenzoylcholine. Interfering coloured substances were eliminated from the *S. alba* crude extract by filtration of 2.50 ml on Sephadex G-25 Medium material in a column (8 × 0.6 cm I.D.). The eluent was water.

TABLE I

SUBSTRATES AND THEIR SOURCES FOR THE KINETIC STUDIES

Measuring wavelengths and corresponding ε values determined in the assay system for substrates and products of the enzyme reactions (pH 6.50).

Substrate and product	*Source*	λ *(nm)**	ε *(l mol^{-1} cm^{-1})*	*Ref.*
Sinapine thiocyanate	*B. campestris*	350	8900	14
Sinapic acid			900	
Isoferuloylcholine acetate	*B. intermedia*	335	14 390	10
Isoferulic acid			3690	
p-Coumaroylcholine acetate	Synthetic	330	13 740	14
p-Coumaric acid			1600	
p-Hydroxybenzoylcholine acetate	Synthetic	270	10 030	14
p-Hydroxybenzoic acid			2330	
Hesperalin acetate	*Hesperis matronalis*	300	5000	10
3,4-Dimethoxybenzoic acid			440	

* λ indicates the wavelength with the greatest difference between substrate and product absorbances.

RESULTS AND DISCUSSION

Determination of enzyme activity at different pH values revealed no distinct optimum, but appreciable activities were found at pH 6–8. Linear relationships between enzyme concentrations and rates of hydrolysis were also found.

Optimal utilization of fast chromatographic techniques calls for a fast spot test. The intensity of the yellow colour in the spot test corresponds to the activity measured in the sinapine assay, except for the aromatic choline esterases from *Barbarea intermedia*. Apparently, the latter enzymes showed a much lower activity for the substrate acetylthiocholine than the other choline esterases. Activity was also revealed by gel electrophoresis.

Fig. 1 shows chromatograms from the development of the FPLC anion-exchange chromatographic techniques used for the purification and characterization of plant aromatic choline esterases. A change in the sodium chloride concentration gradient in the elution buffer (from a to c, Fig. 1) resulted in an efficient separation of the aromatic choline esterase from other seed proteins. The resulting method showed excellent reproducibility, *i.e.*, the esterase activity of a given plant species always appeared at the same sodium chloride concentration in the gradient, the sodium chloride concentration being different for the six species examined (Table II).

In some instances, the flat gradient resulted in a lower recovery of the enzyme than that obtained by use of a gradient from 0 to 1.00 *M* sodium chloride in 20 min. As a compromise between recovery, purification and the need to compare the different enzyme sources, chromatographed under the same conditions, a gradient from 0.10 to 0.35 *M* sodium chloride was used for ion-exchange chromatography. In order to overcome the capacity limitations of the column (total 25 mg of protein) and of the

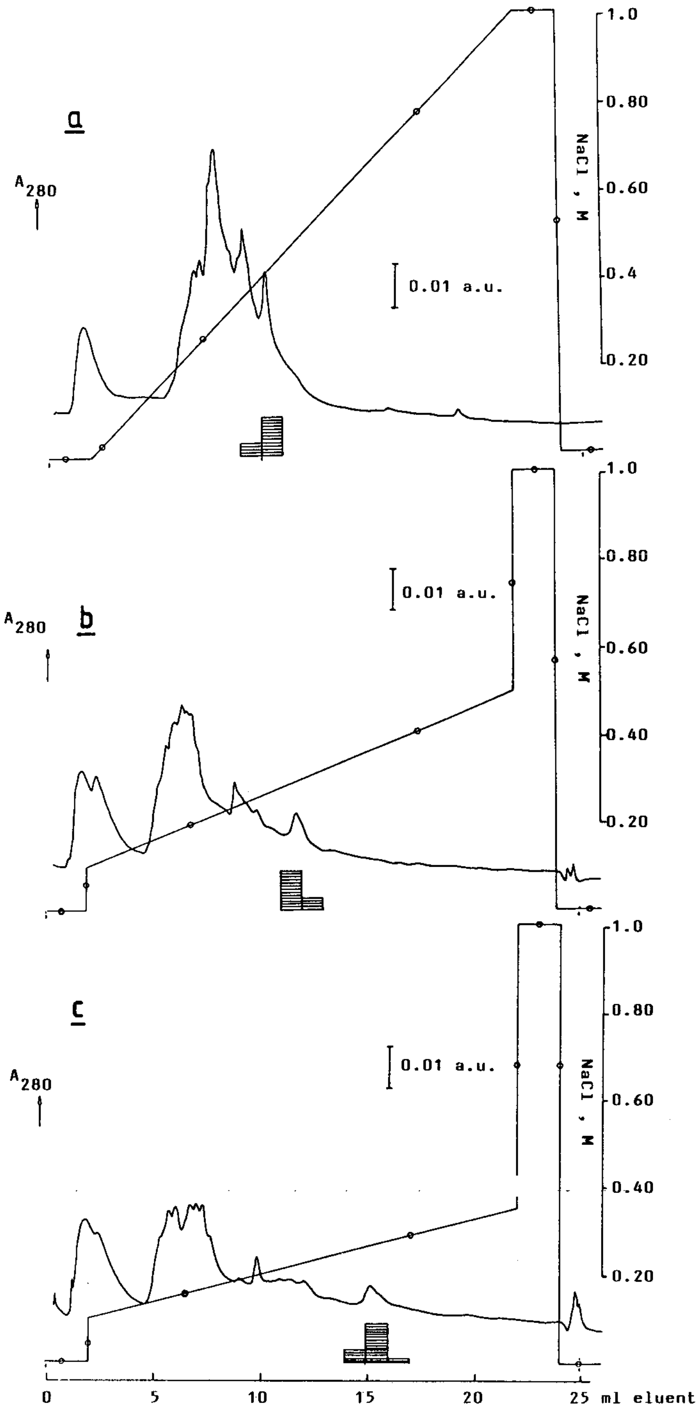

Fig. 1. Optimization of the FPLC ion-exchange isolation of aromatic choline esterase from *Brassica napus* cv. Gulliver. ——, A_{280}; ○—○, concentration of NaCl in eluent. The fractions containing enzyme activity are shaded.

TABLE II

SODIUM CHLORIDE CONCENTRATION IN THE GRADIENT NEEDED TO ELUTE AROMATIC CHOLINE ESTERASE ACTIVITY FROM FPLC ANION-EXCHANGE COLUMN

Plant species	*NaCl (M)*
Barbarea intermedia	0.14–0.17
Brassica nigra	0.14–0.18
Sinapis alba	0.19–0.22
Brassica napus cv. Gulliver	0.23–0.26
Brassica campestris	0.24–0.27
Brassica napus cv. Line	0.24–0.28

injection loop (maximum 1000 μl), successive analyses were performed, and the purified proteins were collected in the same tubes.

The division of the esterases into apparently three groups (Table II) has no explanation yet. The enzymes from *B. napus* and *B. campestris* eluted at the same sodium chloride concentration, whereas the enzyme from *B. nigra* eluted at a much lower concentration. The esterases also behaved differently using chromatofocusing (Fig. 2). The *B. intermedia* esterase was further separated into two isoenzymes. These qualitative differences among the enzymes from the different plants follow differences in the contents of different aromatic choline esters, although the significance thereof is unknown.

Fig. 2 shows selected results obtained by FPLC chromatofocusing of aromatic choline esterases (isoenzymes) from *B. intermedia* (a; fractions 25 and 31) and chromatography after addition of an equal amount of enzyme from *B. campestris*. The enzyme from *B. campestris* (b; fraction 38) is well separated from the two aromatic choline esterases from *B. intermedia* (b; fractions 25 and 31). The technique applied resulted in highly purified enzymes, as revealed by comparison of the curves for protein and enzyme activity (Fig. 2).

Table III shows a comparison of results obtained by purification and characterization of aromatic choline esterases from seedlings of six cruciferous plants.

FPLC ion-exchange chromatography yielded a 3–16-fold purification and nearly quantitative recoveries (67–100%) of the injected activity. This result is in agreement with our previous findings in experiments with the *S. alba* enzyme[11]. The only reported purification of the enzyme is that of Tzagoloff[12], who achieved a 17-fold purification using acetone and ammonium sulphate precipitation of sinapine esterase from *B. hirta* Moench. In newer investigations of the enzyme[13,17] no values for the purification were given.

Ion-exchange chromatography was performed at pH 7.60, which is about three pH units higher than the p*I* of the aromatic choline esterase (Table III). The pH gradient created during FPLC chromatofocusing and the elution of the aromatic choline esterases were reproducible. This allows the determination of relative p*I* values of the enzymes, which can be used in a qualitative comparison of the enzymes from different sources (Fig. 2 and Table III). A comparison of the relative p*I* values obtained by FPLC chromatofocusing and the relative p*I* values obtained by isoelectric focusing has also been performed for other plant enzymes[18].

The results in Table III confirm that the aromatic choline esterases are not

TABLE III

PURIFICATION AND CHARACTERIZATION OF AROMATIC CHOLINE ESTERASES FROM CRUCIFERS

Characterization as revealed from Fig. 2 showing chromatofocusing; this last purification step yielded practically pure enzymes.

Plant species	*Activity (U)*					*Protein (mg)*			
	Crude extract	*$(NH_4)_2SO_4$*	*G-75 filtration*	*FPLC ion exchange (best prep.)*	*FPLC ion exchange (rest)*	*Crude extract*	*$(NH_4)_2SO_4$*	*G-75 filtration*	*FPLC ion exchange (best prep.)**
B. napus cv. Line	2.83	2.25	0.814	0.300	0.036	85.2	36.1	0.956	0.099
B. campestris	1.53	0.996	0.277	0.077	0.027	72.3	34.4	1.16	0.078
B. napus cv. Gulliver	4.84	3.72	1.27	0.199	0.030	48.9	19.0	0.812	0.048
Sinapis alba§§	1.43	1.16	0.326	0.072	0.027	33.3	10.3	0.38	0.024
B. nigra	2.47	1.33	0.406	0.128	0.063	84.2	30.0	0.878	0.216
Barbarea intermedia	0.493	–	0.282	0.162	0.008	25.6	–	1.21	0.180

* FPLC: A_{280} measured.
** Defined as increase in specific activity per step.
*** Percentage activity found of that applied to column.
§ Only relative values estimated from FPLC chromatofocusing.
§§ Molecular weight determined to be 40000 daltons (gel filtration).

TABLE IV

KINETIC PARAMETERS FOR AROMATIC CHOLINE ESTERASES FROM CRUCIFERS

The data were obtained under conditions where no hysteresis effects were observed. None of the examined enzymes showed any appreciable activity towards the substrate 3,4-dimethoxybenzoylcholine (hesperalin). The results for K_m are in μM; the V_{max} values are relative to V_{max} for sinapine.

Plant species	*Substrate*							
	Sinapine		*Isoferuloylcholine*		*p-Coumaroylcholine*		*p-Hydroxybenzoylcholine*	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Barbarea intermedia	23.9	100	23.3	93	3.1	53	n.d.	n.d.
Brassica nigra	6.0	100	1.1	38	4.3	41	5.5	20
Sinapis alba	4.0	100	3.2	30	0.66	42	134	23
Brassica napus cv. Gulliver	11.5	100	3.9	40	3.9	36	52.5	12
Brassica campestris	14.6	100	5.8	39	8.2	30	n.d.	n.d.
Brassica napus cv. Line	10.4	100	n.d.	n.d.	3.7	37	n.d.	n.d.

Specific activity (U/mg protein)				*Purification** (fold per step)*			*Recovery (%)*** [FPLC ion exchange (best prep.)]*	*pI§*
Crude extract	*$(NH_4)_2SO_4$*	*G-75 fil-tration*	*FPLC ion exchange (best prep.)*	*$(NH_4)_2SO_4$*	*G-75 fil-tration*	*FPLC ion exchange (best prep.)*		
0.033	0.062	0.851	3.03	1.9	13.7	15.5	79	4.3
0.021	0.029	0.239	0.992	1.4	8.2	10.7	70	4.3
0.099	0.196	1.56	4.15	2.0	8.0	6.6	67	4.3
0.043	0.113	0.858	3.000	2.6	7.6	5.9	90	4.3
0.029	0.044	0.462	0.593	1.5	10.4	4.7	76	4.4
0.091	–	0.233	0.900	–	–	3.1	100	5.0 and 4

identical, and that different isoenzymes exist in *B. intermedia*. The combination of ion-exchange chromatography and chromatofocusing is valuable for obtaining reliable information about the enzyme activity in various plant species for comparison studies. The stability of the system needs to be high in order to observe minor differences. On the other hand, the same combination of methods can also be used to obtain a larger amount of virtually pure enzyme for more detailed studies, including kinetic studies, for characterization purposes.

Results from enzyme kinetics, based on the use of different substrates, are shown in Table IV. Some combinations of enzyme and substrate concentrations gave rise to hysteresis effects. These combinations were avoided in experiments used to obtain K_m and V_{max} values. The kinetic parameters also confirm that the enzymes for the six sources are different.

It is remarkable that for the enzyme in *S. alba*, *p*-hydroxybenzoylcholine, which is present in the seeds in large amounts, is the poorest substrate. The reason could be that the enzyme isolated is not responsible for the catabolism of *p*-hydroxybenzoylcholine, which occurs much later than the degradation of sinapine in *Sinapis* seedlings[7,10].

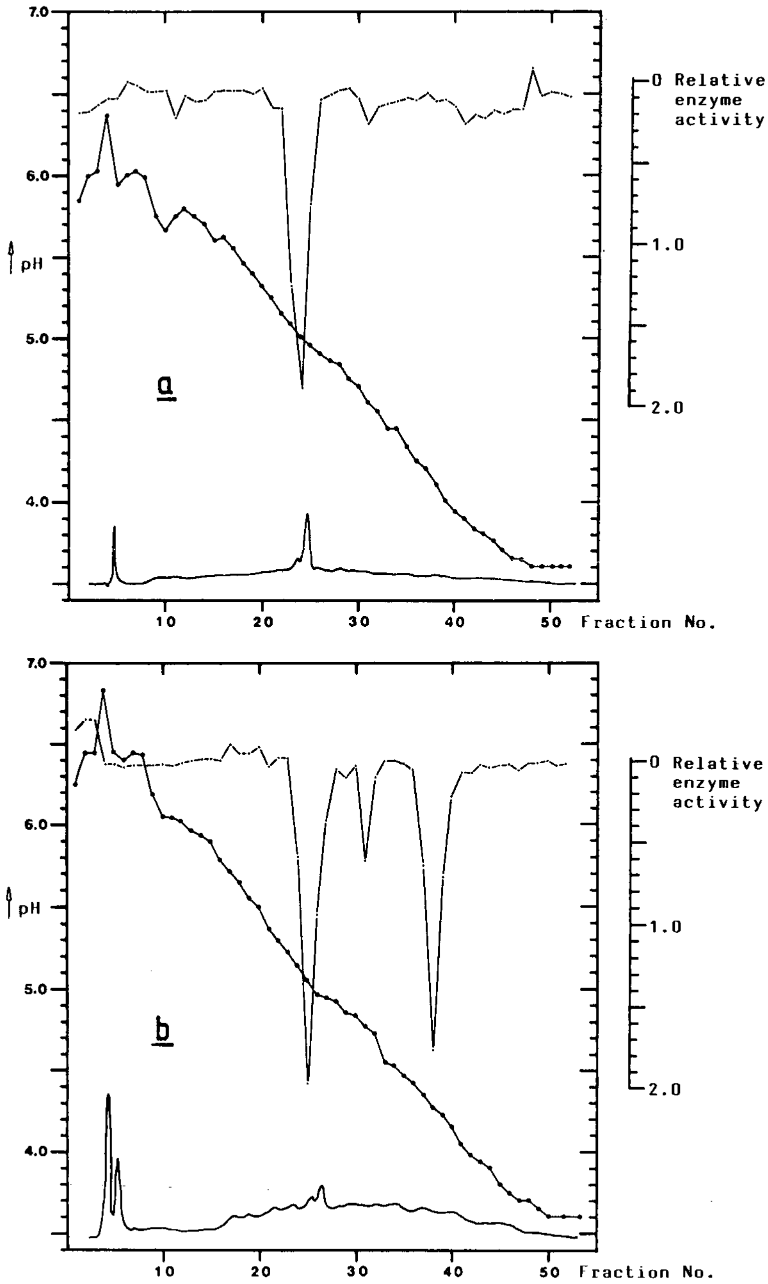

Fig. 2. (a) Chromatofocusing of aromatic choline esterase isoenzymes from *Barbarea intermedia* and (b) chromatography of a mixture with the enzyme from *Brassica campestris*. Solid line (●), pH of the eluates; broken line (●), results from assay; a valley shows activity of aromatic choline esterase. ——, A_{280}.

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PURIFICATION OF SPECIFIC HETEROLOGOUS $F(ab)_2$ FRAGMENTS WITH DEAE-ZETA-PREP® CARTRIDGES FOR ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A simple two-step procedure for purifying $F(ab)_2$ fragments of horse immunoglobulins is described. In the first step, the horse plasma is diluted, made up to 12% (w/v) with ammonium sulphate and digested with pepsin. In the second step, the previously dialyzed solution is chromatographed. Instead of a normal ion-exchange resin, a DEAE-cellulose, covalently linked to a synthetic vinyl polymer, was used (DEAE-Zeta-Prep®). With this assembly it is possible to perform chromatography at a high flow-rate without the problems related to the use of large columns. The yield and purity of the final product are satisfactory. This method has been scaled up for industrial application.

INTRODUCTION

The purification of immunoglobulins or $F(ab)_2$ fragments with ion-exchange resins, in particular with diethylaminoethyl (DEAE-) cellulose, has been described in many papers[1–3]. Although this method gives highly purified immunoglobulins, we have found that its adaptation to an industrial scale is difficult due to the need for large columns and the long adsorption and elution times owing to the compaction of the resins. This problem can be eliminated by using ion-exchange cartridges in which the ion-exchange resin is bound to a solid support that allows high rates of adsorption and elution. In this paper we describe the purification of $F(ab)_2$ fragments of horse immunoglobulins, digested with pepsin, with DEAE-Zeta-Prep® (LKB, Bromma, Sweden) ion-exchange cartridges. As different cartridge sizes are available, this method can easily be scaled up simply by using larger Zeta-Prep 3200 cartridges.

MATERIALS AND METHODS

DEAE-Zeta-Prep cartridges were obtained from LKB. The dimensions were 7 cm × 6.7 cm for Zeta-Prep 250, 24 cm × 12 cm for Zeta-Prep 3200.

Digestion of plasma with pepsin

Plasma from immunized horses was diluted in 2 volumes of water, adjusted to

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pH 4, and digested with 1 g pepsin 2000 FIP (Fédération International Pharmaceutique) U/g (E. Merck, Darmstadt F.R.G.) per litre of undiluted plasma at 42°C for 18 h under continuous agitation in the presence of 12% (w/v) ammonium sulphate[4,5]. The suspension containing $F(ab)_2$ fragments was then filtered through Duplex paper (Galvani, Siena, Italy) and CW 19 filters (Millipore, Bedford, MA, U.S.A.). Digested specific antidiphtheria, antitetanus and anti-snake bite immunoglobulins were studied.

Dialysis

Different ultrafiltration systems were employed depending on the consistency of the digested lots: a Minitan and Pellicon cassette system (Millipore) or two process ultrafiltration cartridges (Millipore). The membranes used had a molecular weight cut-off of 10 000. Dialysis was performed with a constant volume, after the pepsin-digested solution had been concentrated *ca.* 10 times at least 8 volumes of 0.01 *M* phosphate buffer (PB) (pH 6.5) were exchanged. The dialyzate, diluted to 3% in 0.01 *M* PB (pH 6.5), was filtered through CW 19 filters before chromatography.

Chromatography

DEAE-Zeta-Prep cartridges were equilibrated according to the instructions of the manufacturer. The final equilibration buffer was 0.01 *M* PB (pH 6.5). Chromatography was performed at a flow-rate of 40 ml/min for the Model 250 cartridges and 520 ml/min for 3200 cartridges. Under these conditions, $F(ab)_2$ fragments are not bound by DEAE whereas most of the impurities are. The cartridges were therefore eluted at the same flow-rate with 0.01 *M* PB (pH 6.5) in order to recover the $F(ab)_2$, until the eluate had an absorbance of about 0.1 at 280 nm. Then 0.9% sodium chloride was added to the eluate, which was concentrated by ultrafiltration through a Minitan or Pellicon system, until the protein concentration was between 5 and 12%. Purified and concentrated $F(ab)_2$ fragments were filtered in a sterile manner using a Durapore (Millipore) 0.22-μm filter after the addition of 0.3% (w/v) *m*-cresol. Finally the Zeta-Prep cartridges were treated with 1 *M* sodium chloride to wash out the bound impurities, and they were subsequently regenerated according to the instructions of the manufacturer.

Analytical methods

Electrophoresis[6] was performed in 0.5 *M* veronal–EDTA buffer (pH 9.0) on strips of cellulose acetate (14 cm × 5.7 cm), Cellogel (Chermetron Chimica, Milan, Italy), at 200 V for 30 min. Following staining with Coomassie Blue, the percentages of albumin α-, β- and γ-globulins (immunoglobulins) were determined by densitometry. The *in vivo* antibody titer was evaluated in mouse and guinea pigs according to the European Pharmacopoeia[7]. Proteins were determined by the method of Lowry *et al*[8]. Pepsin was determined by counter immunoelectrophoresis (CEP)[9]. The analysis was performed with a plastic plate support, covered with 1% agarose in veronal buffer (pH 8.8) in 3 mm diameter wells, at 50 V/cm for 2 h. Rabbit antipepsin serum was placed in an anodic well and the sample to be analyzed in a separate cathodic well. Another series of wells was filled with antipepsin serum and with standard solutions of pepsin at various dilutions as controls.

High-performance liquid chromatography (HPLC) was performed with a Waters (Millipore, Bedford, MA, U.S.A.) system consisting of a Model 510

solvent-delivery system, a Model 480 absorbance detector and a Model 680 automated gradient controller, with a 600 mm × 7.5 mm TSK G3000SW column (LKB). The eluent was 0.2 *M* phosphate buffer (pH 6.7) containing 0.05% sodium azide. A 10-μl volume sample with a protein content of *ca.* 2% was injected. The toxicity was determined on guinea pigs according to the European Pharmacopoeia[10].

RESULTS

Part of the contaminating proteins in the native plasma were precipitated by addition of 12% ammonium sulphate. The paper- and CW 19-filtered solution was dialyzed to remove the ammonium sulphate and part of the low-molecular-weight proteins, yielding a quantitative recovery of $F(ab)_2$ fragments. Fig. 1 shows the chromatograms of the sample before and after concentration and dialysis. It is obvious that many low-molecular-weight materials were removed. The dialyzed solution containing the $F(ab)_2$ fragments was applied to the ion-exchange cartridges. In a preliminary test it was found that Zeta-Prep 250 cartridges cannot be loaded with amounts of protein exceeding 20 g without loss of large amounts of α-globulins. About 90% of the $F(ab)_2$ was unbound and recovered in 0.01 *M* PB eluate. About 10% of the $F(ab)_2$ was bound to DEAE together with the impurities that are bound under these conditions. The purified solution (90–98%) of $F(ab)_2$, concentrated by ultrafiltration, was free from albumin and pepsin ($< 10^{-4}$ mg/ml) and contained only slight traces of β-globulins. Ion-exchange chromatography increases significantly the purity of the

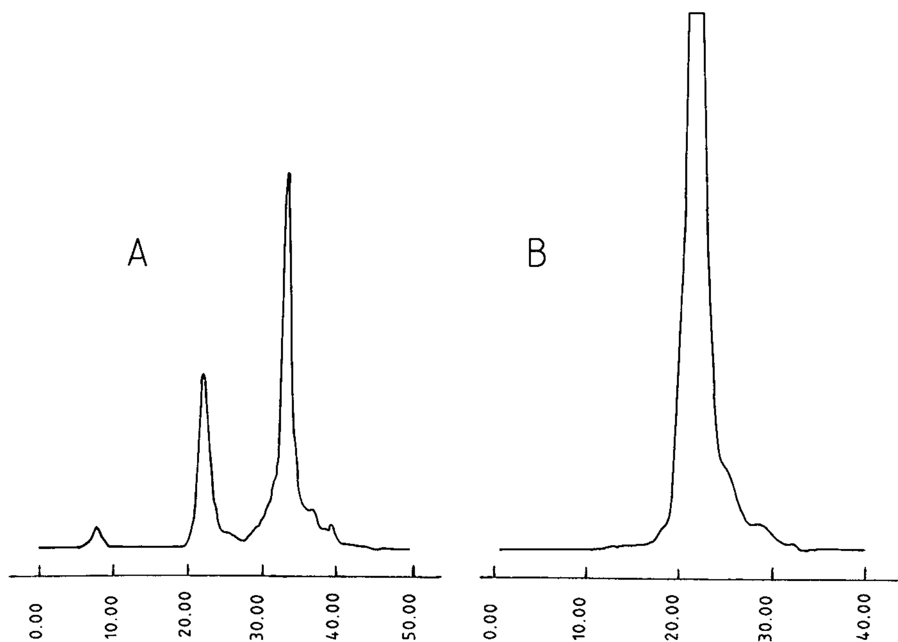

Fig. 1. Chromatograms of (A) digested horse plasma, and (B) digested horse plasma after dialysis. Column: TSK G3000SW (600 mm × 7.5 mm). Eluent: 0.2 *M* phosphate buffer (pH 6.7) containing 0.05% sodium azide. Detection: 280 nm. Time scale in min.

TABLE I

PURIFICATION OF $F(ab)_2$ FRAGMENTS WITH THE ZETA-PREP 250 CARTRIDGE

Chromatography was performed with 0.01 *M* PB (pH 6.5) except where indicated.

Initial plasma			*Final product*							
Lot	*Amount (ml)*	*Titer (I.U./ml)*	*Volume (ml)*	*Protein (g/l)*	*Titer (I.U./ml)*	*Yield (%)*	*Composition (%)**			
							Albumin	*α-Globulins*	*β-Globulins*	*γ-Globulins*
2272	230	450	205	14.8	325	64.4	0	0	2.2	97.8
298A	230	450	170	13.3	350	57.7	0	0	1.5	98.5
298B**	230	450	150	18.7	450	62.5	0	0	5.9	94.1
239A	230	450	180	13.9	350	61.2	0	0	2.5	97.7
239B**	230	450	215	13.7	325	67.5	0	0	6.5	93.5
1791	216	1000	210	13	650	62.3	0	0	1.2	98.8

* By electrophoresis on cellulose acetate.

** 0.03 *M* PB (pH 6.5).

TABLE II

PURIFICATION OF $F(ab)_2$ FRAGMENTS WITH ONE OR MORE ZETA-PREP 3200 CARTRIDGES

Chromatography was performed with 0.01 *M* PB (pH 6.5).

Initial plasma				*Final product*							
Lot	*Amount (l)*	*Titer (I.U./ml)*	*Zeta-Prep cartridges used*	*Volume (l)*	*Protein (g/l)*	*Titer (I.U./ml)*	*Yield (%)*	*Composition (%)**			
								Albumin	*α-Globulins*	*β-Globulins*	*γ-Globulins*
135	3.05	575	1	0.61	70	1750	60.8	0	1.0	8.5	90.5
205	6	209	1	0.7	82.4	739	41.2	0	0.6	11.8	86.8
225	6.3	550	1	1	54	2150	62	0	0	10.3	89.7
110	62	125	3	2.7	128	1500	53.3	0	0	6.4	93.6
610	64	400	3	5.5	75	2500	53	0	2.4	9.6	88
910	53	600	5	7	95	3000	66	0	1.5	5.9	92.6
1510	65	400	4	3.75	131	3500	51.3	0	0.7	5.6	93.7

* By electrophoresis on cellulose acetate.

final product as the solution containing the $F(ab)_2$ fragments has an electrophoretic purity not exceeding 78% before chromatography. The yield of the entire purification process was *ca.* 60–65% (Table I). The losses were due to chromatography, various filtrations and mainly the digestion with pepsin. The yield increased when chromatography was performed with 0.03 *M* PB (pH 6.5), but the purity of the final product was decreased.

Scale-up

For the industrial application of this method we used the larger Zeta-Prep cartridges (3200) allowing work with amounts of horse plasma of 1 l or more. Our results are shown in Table II. A test similar to the one performed earlier with Zeta-Prep 250 allowed us to establish the sample capacity of the cartridge as 220–250 g. It was possible to treat even larger quantities of plasma by arranging many Zeta-Prep 3200 cartridges in parallel. The results are shown in Table II, where the volumes of plasma (up to 60 l) were purified by maintaining a constant protein load for each cartridge.

DISCUSSION

Many purification systems have been used to obtain intact or proteolyzed immunoglobulins suitable for therapeutic use. Ion-exchangers give satisfactory results, but this approach is complicated by the excessive adsorption and elution times needed for processing large volumes of starting materials. The method described combines ultrafiltration with the use of an ion exchanger, supported on a rigid matrix, which allows rapid and simple purification of $F(ab)_2$ fragments. The purity of the product obtained depends on the amount of sample and the dimensions of the cartridge used. The purity of $F(ab)_2$ fragments obtained by using Zeta-Prep 250 is about 98% as determined by electrophoresis. With the larger cartridges we were unable to obtain purity levels higher than 92%. The yields obtained (expressed by *in vivo* antibody titer) were in the range of 50–60%, comparable or superior to those obtained by precipitation with ammonium sulphate. This method can easily be scaled up to an industrial level. The final product is non-toxic and apyrogenic, and thus suitable for human use.

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2nd International Symposium on Preparative and Up-Scale Liquid Chromatography, Baden-Baden, February 1–3, 1988

END OF SYMPOSIUM PAPERS

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journal of chromatography news section

SHORT CONFERENCE REPORT

2nd INTERNATIONAL SYMPOSIUM ON PREPARATIVE AND UP-SCALE LIQUID CHROMATOGRAPHY, BADEN-BADEN, F.R.G., FEBRUARY 1–3, 1988

About 450 participants gathered in a somewhat wintry, wet and windy Baden-Baden for this second symposium in the series. The lectures were very well attended in spite of the warm attractions that give the town its name, and there was active interest in the instrument exhibition, particularly during the coffee and tea breaks, but also throughout the day.

The cognoscenti opined that the papers were of a very high level and that rapid further developments are to be expected in this field.

Fig. 1. A view towards the centre of Baden-Baden taken from the pleasant congress building. No snow, but ample threatening cloud formations. On the ground floor of the building to left of centre may be found a rather expensive but well-appointed bar.

Fig. 2. The symposium chairman, Klaus Unger, looking somewhat questioningly at Hans Poppe who is meanwhile either massaging his right thumb or consulting his watch, or both.

Fig. 3. During the symposium the Pizza Chromatographica was invented at a local pizzeria. Here, the first one is offered reverently to Josef Huber by Peter Hupe. In the background the actual preparer of the newly created delicacy.

Fig. 4. The chairman of the next (third) symposium in the series (1990), Maurice Verzele, of Ghent, in a happy mood at the same event.

Fig. 5. Hyper-modern instrumentation with in the foreground the snappily dressed chairman and Johan Kraak.

(a)

(b)

Fig. 6. The symposium was concluded by an excellent banquet, and dancing. The delightful atmosphere of this closing event can be judged from the happy expressions of (a) Ljudmilla Kolomiets and Douglas Westerlund and (b) the little Chinese lady and Josef Huber.

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