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PREPARATIVE LIQUID CHROMATOGRAPHY WITH ANALYTICAL SEPARATION QUALITY

INTERVAL INJECTION/DISPLACEMENT REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY[a]

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SUMMARY

A method for the enrichment and separation of compounds, called interval injection/displacement reversed-phase high-performance liquid chromatography (HPLC), was applied to several analytical and preparative separations. In principle, the surface of a reversed-phase stationary phase, equilibrated with a weak mobile phase, is "coated" with compounds by stepwise injection of small samples. Distinct time intervals between injections allow the stationary phase to re-equilibrate. In this way, sample enrichment can be achieved in ranges comparable to those in overload elution or displacement HPLC. Sample fractionation proceeds in a similar manner to conventional displacement modes. Also under full mass load conditions, a quality of separation similar to that in analytical elution HPLC is achieved. The results obtained indicate that a very large number of samples can be applied to conventionally sized columns without overload problems, the full mass capacity of a column can be utilized and the method is useful for enriching and separating compounds with a wide range of polarities. Interval injection/displacement reversed-phase HPLC was successfully applied to complex mixtures of natural products and enzyme assay mixtures. Further, this technique is likely to be useful for the analysis of trace compounds and quality control of chemicals. The experiments reported were performed on highly unstable natural products (thiophenic and benzofuran compounds) from *Tagetes* plants.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a highly efficient and versatile method for the analysis of numerous compounds. Analytical separations are typically performed in sample weight ranges below the linear capacity of the stationary phase (less than 1 mg/g of absorbent)[1]. Under these conditions, linear or gradient

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elution causes the movement of a compound through the column without any interference, and compounds are eluted as quasi-Gaussian peaks[1–5]. By using large columns, scale-up of this technique for preparative purposes is possible with maintenance of the high resolution. However, the preparative throughput (yield of pure compound per unit time) is low. Hence expensive stationary phases are only poorly utilized and compounds leave the column in a highly diluted state[1,2,6]. With conventionally sized columns, higher throughputs can be achieved by volume and/or mass overloading (sample weight ranges higher than 1 mg/g of absorbent) in the elution mode or in the recently reviving displacement mode[7–10]. However, the resolution achieved in this way is poor, and for peak cutting one has to make a compromise between high yield (= low purity) and high resolution (= low yield). Sophisticated techniques, *e.g.*, radial or longitudinal column compression, do not substantially resolve this dilemma[11,12]. As stated by Wehrli[13], it seems that the combination of high sample capacity and high resolution cannot be achieved in the same separation.

Another crucial point in preparative work is the necessity to preconcentrate the samples to a minimum volume prior to injection. A severe concentration procedure (often performed by evaporation), particularly of complex mixtures (*e.g.*, crude extracts) or of bulky aqueous solutions (*e.g.*, subjected to trace compound analysis), entails the risk of the formation of insoluble residues, degradation of unstable compounds or artificial product formation[14,15].

Little and Fallick[16] demonstrated the enrichment of aromatic compounds from bulky aqueous solutions on reversed-phase (RP) stationary phases. In this procedure, the solvent of the sample served as the mobile phase during injection. This enrichment technique was subsequently refined but mainly used for analytical purposes[17–21]. Similarly to the displacement mode described above, compounds are enriched at the top of the column under weak solvent conditions and subsequently displaced by a stronger solvent. Further improvements included column-switching techniques and the use of specially coated precolumns[15,17,18]. This method has been successfully applied to the enrichment and separation of phthalic acid derivatives[15,19], various herbicides[17,18], ergot alkaloids and peptide derivatives[20,21]. Nevertheless, each sample volume or mass overloading of the column or interference between the sample solvent and the mobile phase may cause breakthrough[10,17] of the collected material. This phenomenon results in a similar decrease in resolution to that for scaled-up elution or displacement chromatography.

This paper presents some examples of the application of a new preparative technique for enriching and separating compounds, called interval injection/displacement RP-HPLC. As recently described in a short communication[22], this technique allows the application of a very large number of samples to conventionally sized columns, exploiting their full mass capacity. In contrast to the procedures mentioned above, relatively small sample volumes are injected step-by-step, with distinct time intervals between single injections, which allows the stationary phase to re-equilibrate with the weak solvent. Elution and separation of enriched material take place by application of a special displacement procedure which includes a holding phase[19] to maintain reasonable pressure during the fractionation.

Three examples are presented to demonstrate the suitability of the method for the enrichment, separation and purification of (i) a distinct product from a bulky

ethanolic crude plant extract, (ii) enzymatically formed products from buffered, protein-containing solutions and (iii) compounds eluted in bulk from other chromatographic systems (aqueous and organic solvents). Further examples show some empirically estimated limits of the method for maximum sample volume per single injection, the maximum sample mass and the minimum interval time for a given column size. Finally, a performance test was applied to demonstrate the quality of separation attainable with the new method by comparing the results with those obtained using conventional, isocratic and gradient elution modes. All experiments were performed with highly unstable natural products from *Tagetes* plants (Fig.1)[14,23,24].

EXPERIMENTAL

Apparatus and chemicals

The experiments were performed using a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 400 solvent delivery system, equipped with a Rheodyne (Cotati, CA, U.S.A.) 7125 injector (500-μl sample loop), a Kratos SF 769 spectrophotometer (10

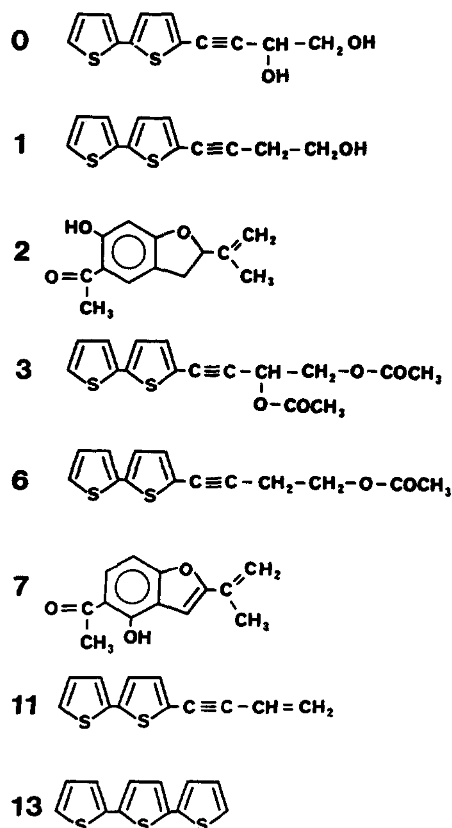

Fig. 1. Natural products extractable from *Tagetes* seedlings[23]. Compound numbers relate to the peaks in Figs. 2–6.

mm pathway cell) and a Spectra-Physics (San Jose, CA, U.S.A.) SP 4100 computing integrator. Columns (250 mm × 4.0, 4.6 or 8.0 mm I.D.) and appropriately sized precolumns were purchased from Knauer (Bad Homburg, F.R.G.). Precolumns were dry-packed with Merck (Darmstadt, F.R.G.) LiChrosorb RP-8 (10 μm). Columns were factory-filled by Knauer with Merck LiChrosorb RP-18 (5 μm) or laboratory-packed (slurry method) with the same material or with Spherisorb (Phase Separations, Queensferry, U.K.) ODS-2 (5 μm). Acetonitrile and water, used as mobile phases, were of HPLC grade (Rathburn, Walkerburn, U.K.). Bithienylbutinene (**11** in Fig. 1) was isolated from *Tagetes* seedlings, as described[23], and α-terthiophene (**13**) was synthesized by Dr. J. Arnason (Ottawa, Canada). Both compounds were used for limit evaluation and performance tests.

Samples

The samples investigated were crude plant extracts, enzyme assay mixtures and affinity chromatography eluates.

Crude plant extracts were prepared from *Tagetes* seedlings by low-temperature extraction[23], concentrated by evaporation and taken up into ethanol.

3,4-Diacetoxybutinylbithiophene:acetate esterase was isolated from *Tagetes* seedlings and was partly purified according to Pensl and Sütfeld[25]. Enzyme assay mixtures (100 μl each) contained 3 nmol of 3,4-diacetoxybutinylbithiophene (dissolved in 10 μl of ethylene glycol monomethyl ether) and the enzyme preparation in 90 μl of potassium phosphate buffer (pH 8.0). The samples were incubated for 10 min and then immediately subjected to HPLC.

A mixture of several compounds from *Tagetes* was immobilized in a Eupergit C (Röhm Pharma, Darmstadt, F.R.G.) column (15 × 8 mm I.D.), equilibrated with 1.0 *M* potassium phosphate buffer (pH 7.5)[26]. Compounds were eluted subsequently with water and with ethylene glycol monomethyl ether (EME) and 100-μl fractions were directly subjected to HPLC.

Mode of interval injection/displacement RP-HPLC and evaluation of limits

Interval injection procedure. Columns of analytical or of semi-preparative size were equilibrated with water–acetonitrile (99:1) using a flow-rate that maintained a constant pressure of about 20 MPa. Under these conditions, any given number of samples could be injected if distinct time intervals were maintained between single injections. The length of the interval time and the maximum applicable sample volume per single injection had to be evaluated empirically for each column size and filling. The maximum applicable sample mass was usually recognizable by a significant increase in pressure after a certain number of injections. After the last injection, enough time was allowed for complete re-equilibration of the column before starting the displacement procedure.

Displacement procedure for separation. After loading the column, the mobile phase was switched to an isocrating holding system[19] containing a strong solvent (acetonitrile) until a constant pressure of about 16 MPa was reached. Previously performed experiments on gradient elution HPLC of thiophenes[23] yielded good results if compounds were eluted in the pressure range 18–10 MPa. Therefore, a similar procedure for displacement was chosen here, using a linear gradient (up to 100% of acetonitrile) and switching to a slightly higher flow-rate to maintain the pressure in this range.

TABLE I

GRADIENT PROFILES EMPLOYED FOR THE SEPARATION AND TRANSFER OF THIOPHENIC COMPOUNDS INTO WATER-FREE MEDIUM

Column: 250 × 4.6 mm I.D. LiChrosorb RP-18 (5 μm) with a precolumn (40 × 4.6 mm I.D.) of LiChrosorb RP-8 (10 μm).

Separation gradient			*Transfer gradient*		
Time (min)	*Flow-rate (ml/min)*	*Water–acetonitrile ratio*	*Time (min)*	*Flow-rate (ml/min)*	*Water–acetonitrile ratio*
0	0.7	99:1	0	0.7	99:1
0.1	0.7	30:70	0.1	0.7	0:100
5.0	0.7	30:70	23.0	1.5	0:100
5.1	1.0	30:70	Indefinite	1.5	0:100
17.0	1.0	0:100			
Indefinite	1.0	0:100			

Displacement procedure for transfer. In order to transfer purified compounds into a water-free medium, material enriched by interval injection was displaced by switching from 1% to 100% of the strong solvent. The expected sudden pressure decrease was compensated for by an increasing flow-rate gradient. Table I gives the gradient profiles employed on a 250 × 4.6 mm I.D. column for the separation and transfer of compounds.

RESULTS

Examples of application

Preparative purification of a product from a crude plant extract. A crude ethanolic extract (5 ml) from *Tagetes* seedlings was applied to an 8.0 mm I.D. RP-18 column by interval injection (10 × 500 μl; interval time, 1 min) and separated as shown in Fig. 2. Fractions of 0.9 ml each were collected and re-chromatographed by analytical HPLC. The re-constructed chromatogram (dilution factor, 1:100) demonstrated the quality of separation of the preparative run. This work was directed to isolate and to purify compound **3**. Therefore, fractions containing **3** were combined (total volume, 4.5 ml) and subjected to a second run under the same conditions (Fig. 3). The peak corresponding to **3** was cut out manually as indicated and this eluate (3 ml) was chromatographed again under transfer gradient conditions (Fig. 4). Cutting out the corresponding peak, as indicated, yielded a product with a purity of nearly 99%, as shown by analytical gradient elution HPLC.

Enrichment and separation of enzyme assay mixtures. Fig. 5 shows the consecutive application of 90 single enzyme assay mixtures (3,4-diacetoxybutinylbithiophene:acetate esterase[25], total volume 9.0 ml, interval time 1 min) to a 4.6 mm I.D. RP-18 column and the separation of products formed (**0** and **3′**, a reaction intermediate[25]) from a given substrate (**3**). The relatively high injection peaks (*cf.*, Figs. 3, 4 and 6–9) suggest that the enzyme protein was eluted during the interval injection period.

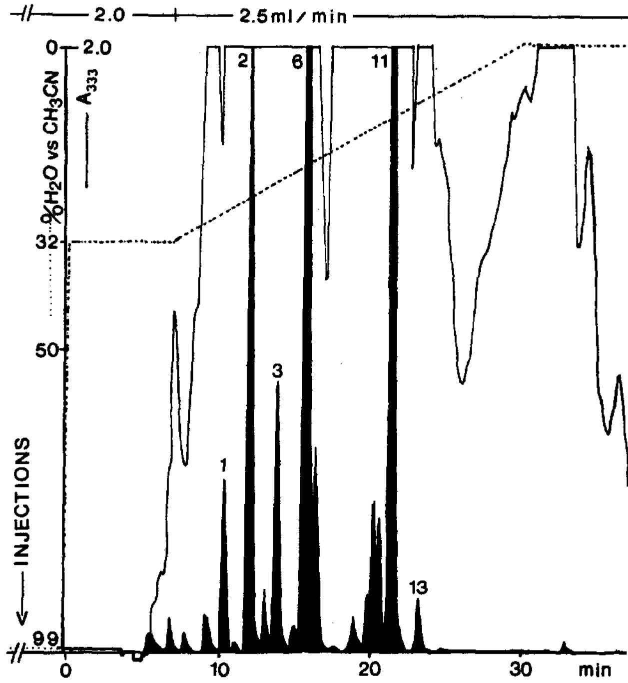

Fig. 2. Fractionation of a crude extract from *Tagetes* seedlings after interval injection. Total extract volume, 5 ml, applied by ten injections of 500 μl each (interval time, 1 min). Solid peaks: re-chromatography of collected fractions by analytical HPLC; dilution factor, 1:100. Column: 250 × 8 mm I.D. LiChrosorb RP-18 (5 μm), with a precolumn (30 × 8 mm I.D.) of LiChrosorb RP-8 (10 μm).

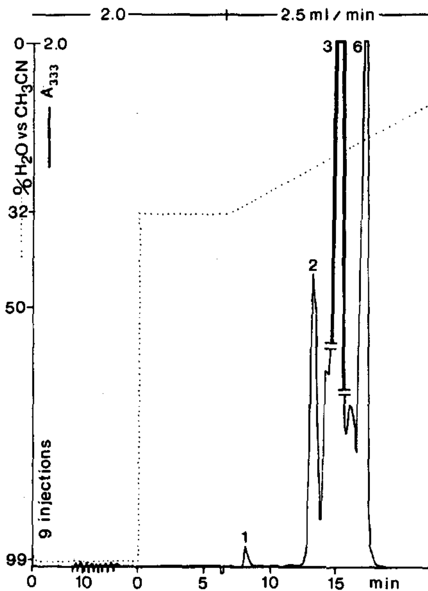

Fig. 3. Interval injection and separation of fractions from the previous run (Fig. 2) containing compound 3. Nine injections of 500 μl each; total volume injected: 4.5 ml. The fraction indicated by the bold line was collected for subsequent transfer into acetonitrile (Fig. 4). Separation conditions as in Fig. 2.

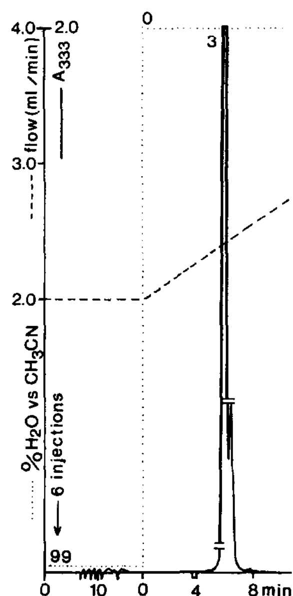

Fig. 4. Interval injection of collected material (Fig. 3) and transfer of compound **3** into acetonitrile. Six injections of 500 μl each; total volume injected: 3.0 ml. The fraction indicated by the bold line was collected for subsequent concentration. Separation conditions as indicated; column and precolumn as described in Fig. 2.

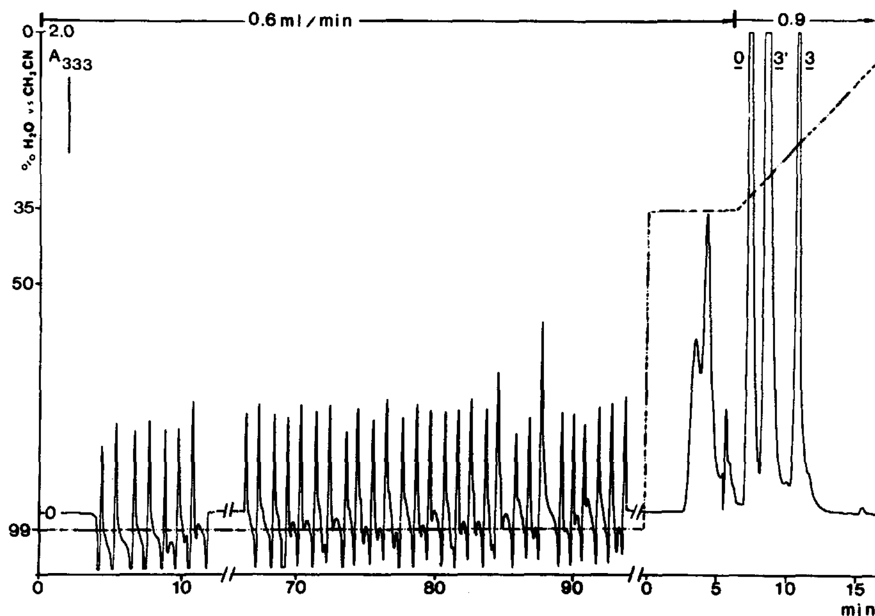

Fig. 5. Enrichment of 90 single enzyme assay mixtures (3,4-diacetoxybutinylbithiophene:acetate esterase[25], total volume 9.0 ml) by interval injection (interval time, 1 min) and separation of products formed (**0** and **3'**) from the substrate (**3**). Compound **3'** supposedly represents 3-hydroxy-4-acetoxybutinylbithiophene[25]. Unnumbered peaks were not further identified. Column: 250 × 4.6 mm I.D. RP-18, 5 μm. Injection: 90 × 100 μl.

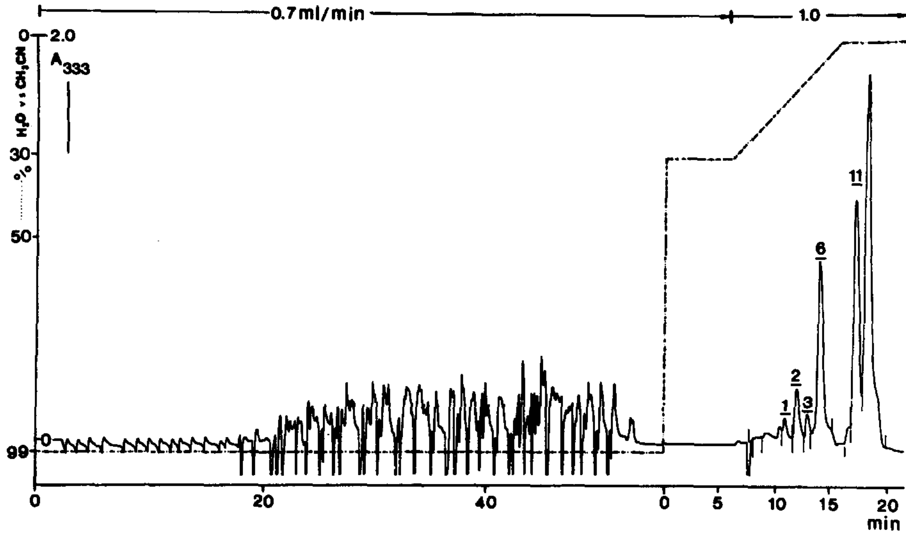

Fig. 6. Enrichment of thiophenic compounds eluted from an affinity chromatography column (Eupergit C). First eluate with 1.5 ml of water, second eluate with 3.2 ml of ethylene glycol monomethyl ether (EME). Interval time, 1 min. Unnumbered peaks were not further identified. Column as in Fig. 5. Injection: 15 × 100 μl eluate (aqueous) + 32 × 100 μl eluate (EME).

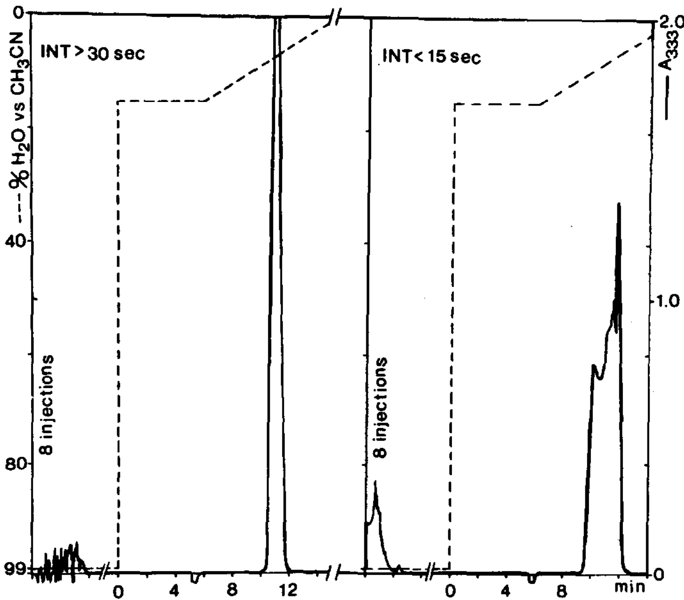

Fig. 7. Influence of interval time on the quality of separation. Sample: α-terthiophene (13) (1.5 mg/ml). Column: 250 × 4.0 mm I.D. LiChrosorb RP-18 (5 μm) with a precolumn (11 × 4.0 mm I.D.) of LiChrosorb RP-8 (10 μm). Flow-rate, 0.7 ml/min during injections, 1.0 ml/min during displacement.

Enrichment and separation of bulk eluates from other chromatographic systems. The suitability of interval injection/displacement RP-HPLC for the enrichment and separation of compounds eluted in bulk volumes and changing solvents is demonstrated in Fig. 6. Several natural compounds of *Tagetes* were immobilized in a Eupergit C column[26] and were subsequently eluted with water and ethylene glycol monomethyl ether. No interference between the different solvents was detectable during the injection time and a reasonable quality of separation was achieved.

Limits of interval injection

The experiments shown in Figs. 7–9 were performed with α-terthiophene (**13**), dissolved in ethylene glycol monomethyl ether, as test substance.

Interval time. Fig. 7 shows that a time interval of at least 30 s has to be maintained between single injections if a column with an I.D. of 4.0 mm and a flow-rate of 0.7 ml/min are used. Shorter intervals, *e.g.*, less than 15 s, yielded poor separations or multiple peaks. Similar results were obtained with columns of larger diameters run with correspondingly higher flow-rates.

Volume per single injection. The maximum applicable volume per single injection is strongly dependent on the I.D. of the column in use. Fig. 8 shows that, for a 4 mm I.D. column, this value ranges between 100 and 200 μl per single injection. For an 8 mm I.D. column, a volume of about 500 μl is still applicable. Too high sample volumes per injection lead to the formation of multiple peaks, similar to those shown in Fig. 7.

Total mass. A 1-ml volume of a saturated solution of α-terthiophene (15 mg) was applied to a 4.0 mm I.D. column in ten injections of 100 μl each. After the tenth injection, a sudden pressure increase was observed, indicating a full mass load.

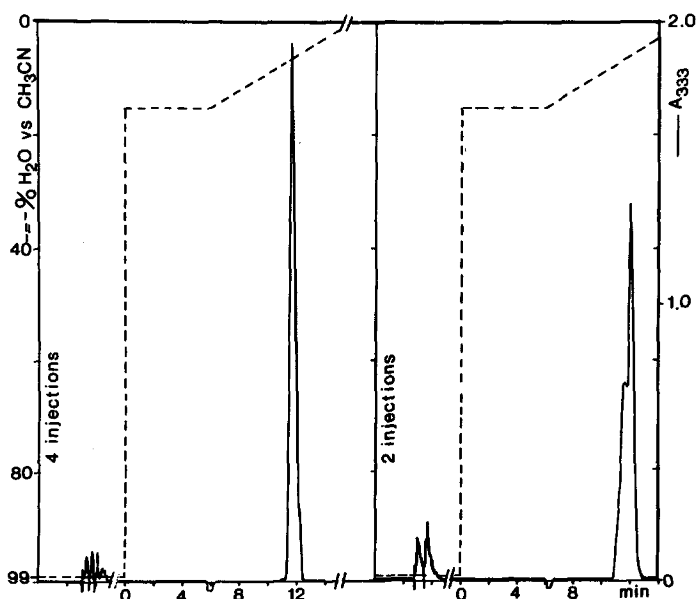

Fig. 8. Influence of the single sample volume on the quality of separation. Sample and separation conditions as in Fig. 7. Left: 4 injections of 100 μl each; right: 2 injections of 200 μl each.

TABLE II

PERFORMANCE TEST USING ISOCRATIC, GRADIENT AND INTERVAL INJECTION/DISPLACEMENT RP-HPLC

Column: 250 × 8 mm I.D. LiChrosorb RP-18 (5 μm) with a precolumn (30 × 8 mm I.D.) of LiChrosorb RP-8 (10 μm). Sample: bithienylbutinene (**11**), 3 mmol/l, 10 μl per injection. Detection, UV (333 nm).

Mode	*Water–acetonitrile ratio*	*Flow-rate (ml/min)*	*Pressure (MPa)*	*Retention time (min)*	*Peak width at half-height (s)*	N[a]
Isocratic	10	3.3	14	6.33	11.6	5900
Isocratic	18	2.8	16	11.06	18.0	7500
Isocratic	32	2.5	19	27.60	49.5	6200
Gradient	32–0 (15 min)	2.0	15–8	16.89	13.9	29 000
Isocratic gradient	32 (8 min) 32–0 (15 min)	2.0 2.5	15–9	20.20	14.6	38 300
Interval injection/displacement	99 (indefinite) 32 (8 min) 32–0 (15 min)	 2.0 2.5	22–9	20.39	20.6	19 600

[a] N, theoretical plate number, calculated by using retention times and peak widths at half-heights[1,27].

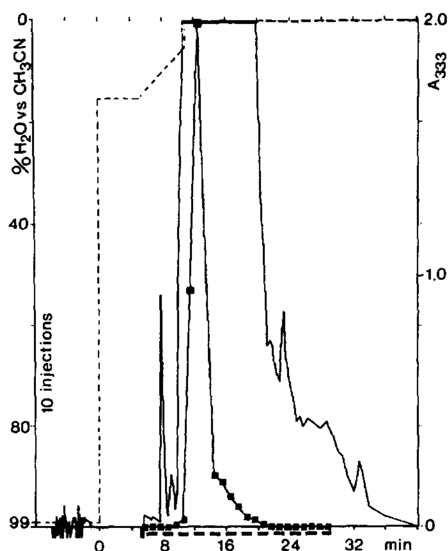

Fig. 9. Interval injection/displacement RP-HPLC under full mass-load conditions. Sample, α-terthiophene (13) (15 mg/ml). Separation conditions as in Fig. 7. Fractions were collected as indicated by the black and white bars and re-chromatographed by analytical HPLC at a dilution of 1:100 (■). Ten injections of 100 μl each.

Fractions were collected as indicated in Fig. 9 and re-chromatographed (dilution, 1:100) by analytical HPLC. The reconstructed chromatogram demonstrated the quality of separation in the preparative run. Moreover, the preparative chromatogram gave evidence of the presence of some impurities that could not be observed before.

Performance test

Identical samples of bithienylbutinene (11) were chromatographed under various isocratic, gradient and isocratic gradient conditions and also under the conditions of interval injection/displacement HPLC. Table II shows that, according to the calculated theoretical plate numbers, the interval injection method yielded a reasonably high resolution in comparison with the conventional linear and gradient elution methods, which were run under similar mobile phase conditions.

DISCUSSION

Interval injection/displacement RP-HPLC has been shown to be a powerful method for the enrichment and separation of natural plant compounds. It is probably also applicable to a wide range of other natural or synthetic products of similar polarities. Compounds with higher polarities can probably be enriched by modifying the weak solvent on injection, *e.g.*, by acidification or by addition of ion-pair reagents.

With interval injection, concentration of extracts prior to HPLC injection becomes superfluous and hence some new compounds may now be detected that would have been degraded because of severe pretreatment procedures[14]. The results obtained indicate that, with this method, column mass loads can be achieved in ranges

comparable to those in overload elution or displacement modes[1,2,6]. However, employing the interval injection mode, a quality of separation is achieved that resembles analytical runs. It may be assumed that, during interval injections, compounds do not invade the stationary phase but coat the surface of the column as a thin layer. Full mass load is reached if this layer becomes impermeable to the mobile phase, as indicated by a sudden increase in pressure.

Obviously, interval injection/displacement RP-HPLC is able to combine a high preparative throughput with high resolution, in contrast to previous experience[13]. Further, the method is applicable to trace compound analysis and chemical quality control. The new possibility of enriching compounds and transferring them into a water-free medium opens up other prospects for application. For enzymological research, the method has several advantages, *e.g.*, enzyme assay mixtures can be injected without stopping the reaction by treatment with acid, alkali or organic reagents for protein precipitation. The results obtained (Fig. 5) suggest that the native protein is eluted from the column during the interval injections. Because of the possibility of injecting numerous assay mixtures, extremely low enzyme activities can be detected. However, one may argue that a similar enrichment and separation effect could be achieved if the whole of the aqueous solution were to be applied in one step, *e.g.*, by pumping it through the system or by using appropriately sized sample loops as previously described[16–21]. However, in contrast to the disadvantages of these techniques, interval injection does not involve the risk of damage to the solvent delivery system by microparticulate material or by aggressive buffer ions which are often present in enzyme assay mixtures. The size of the sample loop becomes unimportant and hence the number and size of single injections are completely variable.

Interval injection works well if some precautions are taken. Aqueous and organic sample solvents may be injected when they are soluble in the mobile phase (*cf.*, Fig. 6). The length of the interval times should be adapted to the column size (I.D.) and to the sample solvent used in order to prevent compounds from invading the column. The maximum volume per single injection seems to be an important factor, which must be determined and adapted to each individual column size and filling, otherwise, breakthrough[10,17] of collected material will occur, leading to poor separations. Further experiments to apply this method to other compounds and stationary phases are in progress.

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REFERENCES

1 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.
2 M. Verzele and C. Dewaele, *Preparative High Performance Liquid Chromatography —A Practical Guideline*, TEC, Ghent, 1986.
3 R. M. McCormick and B. L. Karger, *J. Chromatogr.*, 199 (1980) 259.

4 F. Riedo and E. sz. Kováts, *J. Chromatogr.*, 239 (1982) 1.
5 Cs. Horváth, J. Frenz and Z. El Rassi, *J. Chromatogr.*, 255 (1983) 273.
6 G. Guiochon and A. Katti, *Chromatographia,* 24 (1987) 165.
7 Cs. Horváth, A. Nahum and J. H. Frenz, *J. Chromatogr.*, 218 (1981) 365.
8 M. Verzele, C. Dewaele, J. van Dijck and D. van Haver, *J. Chromatogr.*, 249 (1982) 231.
9 J. Frenz, Ph. van der Schriek and Cs. Horváth, *J. Chromatogr.*, 330 (1985) 1.
10 V. R. Meyer, *J. Chromatogr.*, 316 (1984) 113.
11 G. Cretier and J. L. Rocca, *Chromatographia,* 16 (1982) 32.
12 E. Bayer and H.-P. Seelmann-Eggebert, *Chromatographia,* 18 (1984) 65.
13 A. Wehrli, *Fresenius Z. Anal. Chem.*, 277 (1975) 289.
14 R. Sütfeld and H. Breteler, *Proc. Int. Conf. NOARC's,* Elsevier, Amsterdam, in press.
15 H. P. M. van Vliet, T. C. Bootsman, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 185 (1979) 483.
16 J. N. Little and G. J. Fallick, *J. Chromatogr.*, 112 (1975) 389.
17 C. E. Goewie, P. Kwakman, R. W. Frei, U. A. Th. Brinkman, W. Maasfeld, T. Seshadri and A. Kettrup, *J. Chromatogr.*, 284 (1984) 73.
18 M. Åkerblom, *J. Chromatogr.*, 319 (1985) 427.
19 A. Otsuki, *J. Chromatogr.*, 133 (1977) 402.
20 P. Schauwecker, R. W. Frei and F. Erni, *J. Chromatogr.*, 136 (1977) 63.
21 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
22 R. Sütfeld, *Fresenius Z. Anal. Chem.*, 330 (1988) 395.
23 R. Sütfeld, *Modern Methods of Plant Analysis*, Vol. 5, Springer, Berlin, 1987, p. 104.
24 R. Sütfeld, *Planta*, 156 (1982) 536.
25 R. Pensl and R. Sütfeld, *Z. Naturforsch., C.*, 40 (1985) 3.
26 R. Sütfeld, in preparation.
27 A. T. James and A. J. P. Martin, *Analyst (London)*, 77 (1952) 115.