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TWO-DIMENSIONAL COLUMN LIQUID CHROMATOGRAPHIC TECHNIQUE FOR RESOLUTION OF COMPLEX MIXTURES

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SUMMARY

The improvement of a given high-performance liquid chromatographic (HPLC) system (better selectivity, α; more theoretical plates, N) in order to be able to handle complex separation problems is often tedious and costly. The possible alternatives may be gradient elution or column-switching techniques, but they do not always lead to the desired effects. A two-dimensional HPLC separation technique (similar to the well known two-dimensional thin-layer chromatography), which permits the use of two different separation mechanisms, would often be highly desirable.

The possibilities of such an HPLC device have been investigated. It consists of a first column filled with gel permeation chromatographic (GPC) material, which is coupled via a loop injection device with a second column containing reversed-phase material. Such a device permits the on-line collection of fractions from the first column and direct injection into the second chromatographic system. The size of the fractions in the first separation is flexible and is controlled by the frequency of injection on to the second column.

By exploiting pre-concentration phenomena on the second, reversed-phase column, fractions of several millilitres can be collected and injected without a loss in resolution in the following chromatographic step. The second separation (second dimension) can even be coupled with a solvent gradient for further separation enhancement.

The combination of GPC and reversed-phase chromatography was used for the separation of complex plant extracts. Seven fractions with volumes of about 1.5 ml per fraction were subjected to the reversed-phase separation in the second dimension. The sequences for fraction collection and re-injection are automatically controlled. The aim of this investigation was to obtain a better separation and more information on a mixture of senna glycosides.

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INTRODUCTION

Although high-performance liquid chromatography (HPLC) is a powerful separation technique, it frequently approaches to its limits when applied to complex mixtures in a complex matrix. Selectivity-enhancing steps such as the use of more efficient columns, gradients or derivatization techniques[1] can deal with some of these complex systems, but the first two will remain one-dimensional in their possibilities. This leads to the idea of using two-dimensional or even multi-dimensional techniques. Two-dimensional planar chromatographic procedures [thin-layer (TLC) and paper chromatography (PC)] are well known and have been used extensively in medicinal and biological analyses, where the handling of complex samples is a daily routine. These two-dimensional and multi-dimensional techniques for amino acids and peptides have been reviewed by Pataki[2], who also discussed the combination of TLC–electrophoresis (first dimension) and regular TLC (second dimension) for such systems.

Two-dimensional chromatography combining on-line column chromatography with planar techniques has also been reported for classical liquid column chromatography[2,3]. An on-line combination of gas chromatography (GC) with TLC has been described[4] and a review of this topic was published by Janák[5].

One could regard combined gas chromatography–mass spectrometry (GC–MS) as a modern two-dimensional approach. A modification of this idea is the coupling of GC with plasma chromatography[6].

Another two-dimensional coupling device in GC was recently described by Aue[7], who directed selected fractions from a first column to a low-energy plasma reaction chamber. The reaction products were then flushed by argon carrier gas through a second column with different separation characteristics.

Two-dimensional techniques using columns in both dimensions in an on-line fashion have not yet been used, probably owing to technical difficulties. Obviously, we are not considering the manual collection of fractions and a re-run of the fraction on another column as a modern two-dimensional approach. Column-switching techniques reported for both GC[8] and HPLC[9,10] are a step in this direction but are not really two-dimensional as the mobile phase is the same, the column materials differ only slightly and the same separation principle is used.

In this paper, we discuss genuine column coupling as a two-dimensional HPLC approach. It should be emphasized, however, that a direct comparison of this approach to two-dimensional TLC is not justified as the same component may appear in several chromatograms in the second run. On the other hand, we are not only introducing a new solvent system for the second dimension but also a new stationary phase. This study was prompted by the complexicity of recent problems involving the resolution of mixtures from plant extracts[11]. A favourable starting point was the discovery of pre-concentration phenomena on injecting aqueous samples of relatively non-polar substances on reversed-phase columns[12,13]. This permits the transfer of aqueous fractions from an ion-exchange or gel permeation chromatographic (GPC) column to a reversed-phase column (second dimension) with little or no band broadening[13].

DESCRIPTION OF THE SYSTEM

Basic considerations

The power of a combination of two or more techniques to give a two- or multi-dimensional method is considerably enhanced if the combination consists of completely independent techniques. Information theory[14] shows that the total information consists of the sum of the individual information of each individual technique minus the synentropy (cross-information):

$$J_{tot} = \sum_{k=1}^{m} J_k - \sum_{i,j=1}^{m} T_{ij} \quad (1)$$

where

J_{tot} = total information of the m-dimensional technique;
J_k = information of the single technique;
T_{ij} = synentropy (cross-information) of techniques i and j.

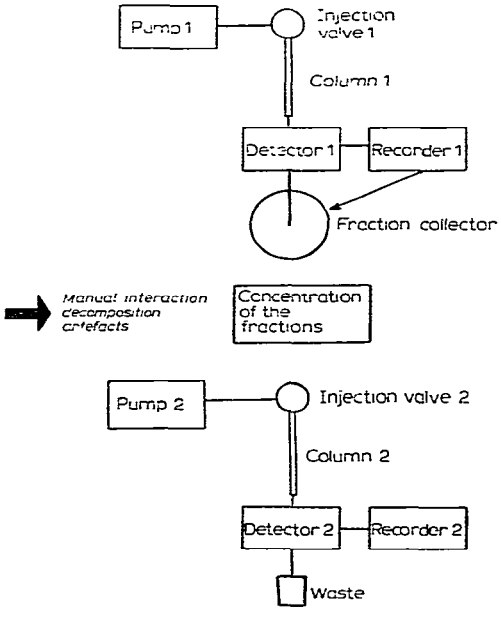
Keeping the synentropies as low as possible is therefore a prime aim in combinations to give a multi-dimensional method. As an example, the coupling of GC and MS (*i.e.*, a separation technique and a spectroscopic technique) yields a very small synentropy and is therefore, from the point of view of information theory, a good coupling method. For two-dimensional TLC, on the other hand, the synentropy is large as the same adsorbent layer must be used in both dimensions. For multi-dimensional HPLC, it is possible in principle to combine different separation principles, which means that the synentropy is relatively small.

The combination of GPC (separation on the basis of molecular size) with reversed-phase chromatography (RPC) (separation of compounds with different hydrophobicities) results in a negligible synentropy.

Instrumental aspects

The classical approach to multi-dimensional column chromatography in an off-line arrangement by collection of fractions, concentration of the fractions and re-introduction on to a different column has been practised for many decades and could be called an off-line two-dimensional technique. As can be seen in Fig. 1, this approach is complicated and tedious. The most time-consuming and difficult step is the sample concentration step needed prior to re-injection. Another important disadvantage is the possibility of the formation of artefacts due to decomposition of sensitive substances in the interface step.

The adaptation of the use of modern pre-concentration techniques[12,13] can lead to an interface that eliminates this time-consuming step and the adverse effects resulting from it. The pre-concentration technique is actually based on the fact that when relatively non-polar substances from an aqueous solution are injected into a reversed-phase column, the substances will be retained in a very narrow zone on top of the column. Subsequent elution with a less polar solvent will start the separation of this pre-concentrated zone with essentially no further band broadening due to the injection volume. This principle was first reported by Little and Failick[15] and has been systematically explored for organic species[12,13] and anions[16]. Similar phenomena could be exploited with adsorption chromatographic systems (hydrophilic surfaces) using suitably altered polarity conditions.



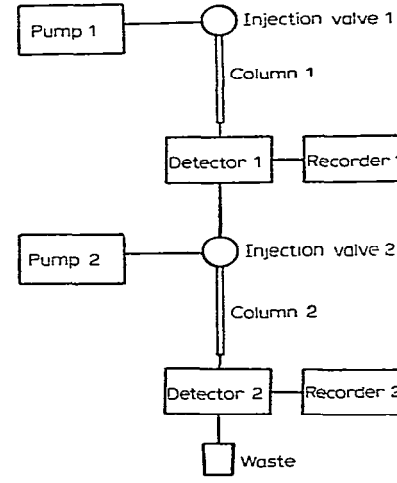


Fig. 1. Flow chart of the off-line procedure for the combination of two separation systems.
Fig. 2. Schematic diagram of on-line two-dimensional HPLC.

For an exploitation of this phenomenon in our two-dimensional apparatus, it is therefore essential that the mobile phase in the first dimension is suitable for preconcentration on top of the second column, *i.e.*, an aqueous or at least highly polar solvent system in the first dimension if the second dimension is to be RPC or a non-polar solvent if adsorption chromatography is planned for the second dimension. The combination of GPC with RPC, as used as an example in this work, is well suited for this purpose.

Another condition is a relatively slow flow-rate for the first dimension, in order to give sufficient time for the analysis of the fractions in the second dimension.

Fig. 2 shows a typical apparatus for on-line operation of such a two-dimensional technique. The fraction collection and sample reduction step (interface) depicted in Fig. 1 are now replaced with a sampling loop, which is filled by the first eluent after passing through column 1 and an optimal detector 1. The use of pneumatic operation for the injection loop 1 permits a fully automated two-dimensional separation coupled with the obvious advantage of automated systems such as time saving, better reliability and reproducibility of the technique. The size of the injection loop 2 is determined from the following relationship:

$$l_2 \geqslant f_1 t_2 \tag{2}$$

where

l_2 = size of the injection loop of the HPLC system in the second dimension (ml);
f_1 = flow-rate of the HPLC system in the first dimension (ml/min);
t_2 = analysis time of the HPLC system in the second dimension (min).

For an on-line operation of the system, a special eight-port loop valve such as that depicted in Fig. 3 can be used. There is also the possibility of operating in a stop-flow mode or of accepting a loss of eluate in the first chromatographic step. Such a loss can be calculated from the rinsing time, t_{r2}, of loop 2 (set to the inject position), the flow-rate 2 and the size of loop 2 as follows:

$$t_{r2} = \frac{l_2}{f_2} \tag{3}$$

During the rinsing time, t_{r2}, the eluate from column 1 goes to waste. The resulting loss, L, is therefore

$$L = f_1 \, t_{r2} = f_1 \cdot \frac{l_2}{f_2} \tag{4}$$

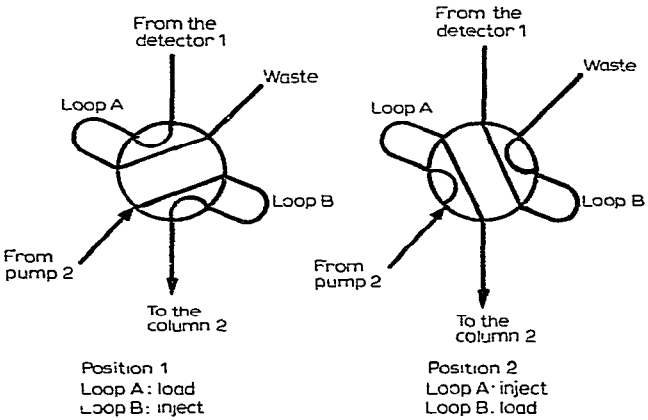


Fig. 3. Schematic diagram of an eight-port dual-loop injection valve for injection into the second column without loss.

where f_2 is the flow-rate of the HPLC system in the second dimension. The relative loss, L_{rel}, is therefore

$$L_{rel} = \frac{L}{l_2} = \frac{f_1}{f_2} \tag{5}$$

For the present study the combination of the slow GPC (*e.g.*, 0.02 ml/min) with the rapid RPC (*e.g.*, 2 ml/min) yields only a small loss (*ca.* 1%). In any event, the time required for the separation in the second dimension dictates the low flow-rate, f_1, of the first dimension.

EXPERIMENTAL AND RESULTS

A schematic diagram of the apparatus is shown in Fig. 4. The first dimension is GPC and the second dimension RPC with a step gradient[11]. Senna glycosides from a complex plant extract were investigated. The senna glycoside solution is injected via loop 1. The separation is carried out with an aqueous buffer solution of pH 7 on

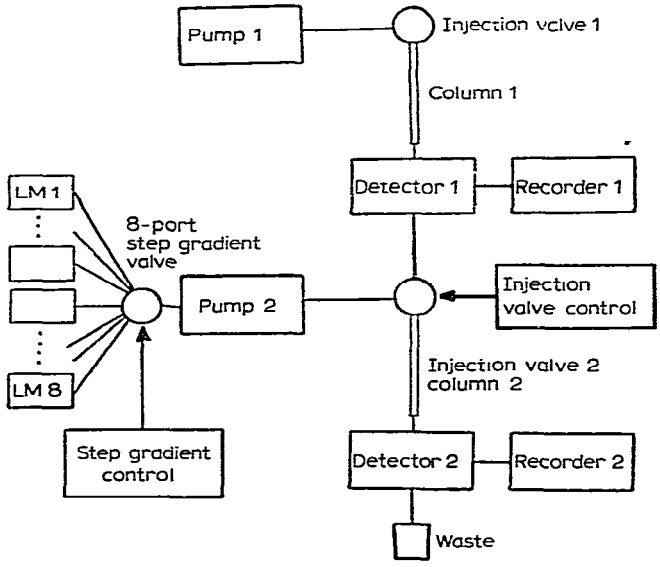


Fig. 4. Schematic diagram of on-line two-dimensional HPLC with step-gradient elution and automatic injection control of loop 2. Pump 1: Lewa Model FL 1 (Lewa, Herbert Ott AG, Leonberg Stuttgart, G.F.R.). Injection valve 1: Valco loop, 7000 p.s.i., 50 μl (Valco Instruments, Houston, Texas, U.S.A.). Column 1: stainless steel, 200 × 0.4 cm, filled with CPG (controlled pore glass), 200–400 mesh, 88A/113/A 170A/240A (Electro-Nucleonics, Fairfield, N.J., U.S.A.). Detector 1: LC 55 UV detector (Perkin-Elmer, Norwalk, Conn., U.S.A.). Recorder 1: W + W Model 600 recorder (Kontron, Zürich, Switzerland). Injection valve 2: Valco loop, 7000 p.s.i., 1.777 ml. Control unit for the injection valve: home-made, two type RDF time relays (Summerer, Zürich, Switzerland). Pump 2: Altex Model 100 (Altex, Berkeley, Calif., U.S.A.). Step gradient valve: Labotron eight-port valve, No. 2581 (Kontron). Step gradient control unit: home-made, RS 21 × PG time relays (Comateletric, Worb, Switzerland). Column 2: stainless steel, 25 × 0.4 cm, filled with Nucleosil C_{18} reversed-phase material, 5 μm (Machery, Nagel & Co., Düren, G.F.R.). Detector 2: L 55 UV detector (Perkin-Elmer). Recorder 2: W + W Model 600 recorder (Kontron).

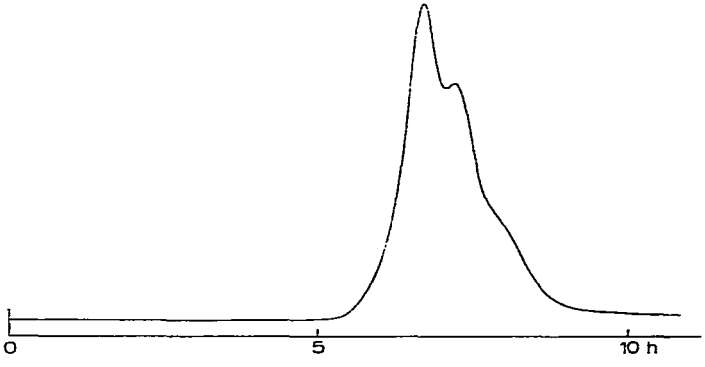


Fig. 5. GPC separation of a senna glycoside extract (trace from recorder 1 in Fig. 4). Mobile phase, buffer, pH 6 (Titrisol; Merck, Darmstadt, G.F.R.); flow-rate, 1.2 ml/h; detection, UV (254 nm). Chromatographic equipment as described in Fig. 4.

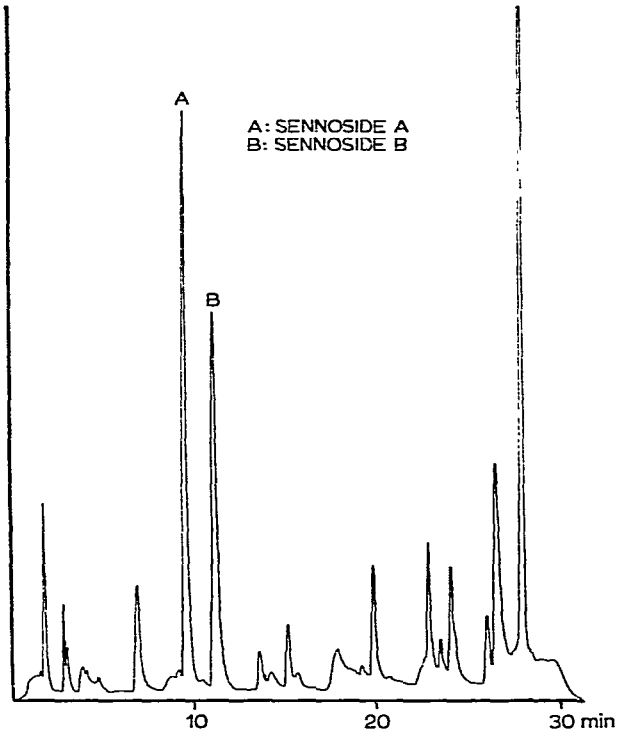


Fig. 6. RPC separation of the same senna glycoside extract as in Fig. 4. Mobile phase, seven steps of acetonitrile–0.01 *N* sodium hydrogen carbonate in water; flow-rate, 2 ml/min; detection, UV (254 nm). The gradient steps were as follows:

step 1	1.5%		before injection
step 2	4.2%		5 min
step 3	6%	acetonitrile	6 min 20 sec
step 4	7.5%	in 0.01 *N*	3 min 30 sec
step 5	10%	$NaHCO_3$–water	5 min
step 6	12.5%		6 min 20 sec
step 7	50%		2 min 30 sec

Chromatographic equipment as described in Fig. 4. Peaks: A, sennoside A; B, sennoside B.

a GPC column at a low flow-rate. Each fraction is collected and injected via the loop, concentrated on top of the column and separated on a reversed-phase column.

Generally, the flow of pump 1 (GPC system) is adjusted such that loop 2 (1.777 ml) is just filled during the separation time (*ca.* 1.5 ml). The flow of the reversed-phase system (pump 2) is such that complete flushing of loop 2 is possible within the injection time.

The complex chromatograms that are obtained with such a senna glycoside extract when only one dimension is used are shown in Fig. 5 for the GPC run and Fig. 6 for the step-gradient RPC run[11].

Reversed-phase chromatograms of seven fractions from the GPC run as obtained with the apparatus shown in Fig. 4 by using a step gradient are presented in Fig. 7. The value of the two-dimensional approach is clear: the originally very complex chromatograms (Figs. 5 and 6) are now subdivided into a set of seven simpler and better resolved chromatograms.

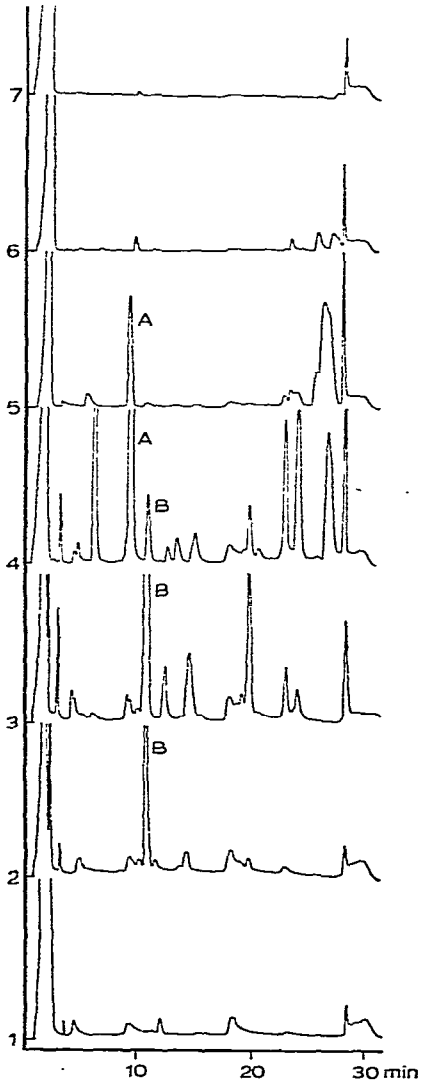


Fig. 7. RPC runs of seven fractions from the GPC run of the same senna glycoside extract as in Fig. 6. Peaks: A, sennoside A; B, sennoside B.

CONCLUSIONS

It has been demonstrated that two-dimensional HPLC is a feasible approach for the separation of major and minor components in complex mixtures for better identification. Such a system can be fully automated, which ensures better reliability of operation and reproducibility of the data. Coupling with computer systems will also permit the chromatographic data generated by such a technique to be handled efficiently. This would lead to a three-dimensional presentation of the chromatographic output for easier interpretation[17].

Although in this study the principle has been demonstrated only with a combination of GPC and RPC, this could easily be expanded to other combinations such as ion exchange–RPC; affinity chromatography–RPC and GPC or affinity chromatography–adsorption chromatography. The only strict condition to be observed is that in the second dimension the pre-concentration effect should be produced. Otherwise, band broadening phenomena will eradicate any increase in selectivity in the two-dimensional approach.

A further development of this philosophy would be a move to multi-dimensional techniques, *i.e.*, more than two columns with different separation principles. Other multi-dimensional aspects could be introduced by using different detection modes for each dimension. Although in this sudy single-wavelength UV detectors were used, this could be extended to a combination of different detectors such as UV–fluorescence, UV–mass spectroscopic or electroanalytical detectors, possibly still further coupled to chemical derivatization after the first or subsequent separation step.

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