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NEW PRINCIPLE OF SAMPLE INTRODUCTION INTEGRATED WITH MOBILE PHASE DELIVERY FOR MICRO-COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

The possibilities and limitations of a liquid micro-chromatograph with integrated sample introduction and mobile phase delivery systems are considered. Basic values of chromatographic constants such as capacity ratio and number of theoretical plates are expressed as a function of a defined and limited retention volume.

A new type of the liquid micro-chromatograph is described. The sample introduction system is integrated with a mobile phase delivery system in one instrumental section. A gradient of the mobile phase can be generated in the device. The new principle is applicable as a portable, fully automated analyser. Volumes of 0.01–500 μl can be injected. A controlled sample introduction velocity makes it possible to generate extremely small sample dispersions. A column efficiency of near to theoretical levels can be achieved with an amperometric detector. Both trace analyses and rapid chromatographic separations are demonstrated.

INTRODUCTION

Miniaturization leads to a number of advantages but also to technical problems, as is well known. However, it also forms a good basis for the design of modern, fully automated and tunable analysers applicable not only to large series of analyses at different locations but also to series of analyses with great variability of the chromatographic systems.

The term "analyser tunability" implies the possibility of mobile phase selection and its rapid change or the use of a gradient of mobile phase composition. Also, a change in the sorption properties of the stationary phase must be possible, *e.g.*, by means of a dynamically generated sorption layer of a modifier on an adsorbent. At the same time, it will be necessary to ensure also the possibility of selecting the amount of sample injected over a sufficiently wide range without any requirement for mechanical changes to the analyser.

A chromatograph[1] based on miniaturization of individual elements such as a column and a detector has been designed and tested. The apparatus is equipped with a device combining in one element injection of the sample and delivery of the mobile

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phase, the composition of which may change for different kinds of analyses. The device also serves as a generator of the mobile phase gradient.

Assuming that it is possible to work with small retention volumes, columns of sufficiently small diameters were used. In the apparatus designed the mobile phase does not flow through the column in the period between individual analyses. Further, the apparatus also has simplified pumping and injection elements of the chromatograph and a substantially reduced extra-column volume.

The design simplifies the gradient technique within the dimensions of the micro-column chromatograph. The injection system allows the sample injection volume to be changed smoothly in the range 0.01 μl–0.1 ml without touching the fixedly assembled elements. Consequently, it is possible to achieve good results in both mass trace analysis and concentration trace analysis. It has been verified that with the chromatograph arranged in this way the service life of the micro-column is substantially longer than in chromatographs of standard design.

Advantages of the apparatus, in addition to increased speed of analysis resulting from the properties of the chromatographic system, include versatility, mobility and reliability.

The preparation of the sample and of a suitable mobile phase leads to improved chemical flexibility, allowing in many instances the analysis to be simplified and the certainty of identification and the sensitivity of the analysis to be increased.

ROLE OF THE RETENTION VOLUME

A decrease in the column diameter leads to a decrease in the mass of the sorbent used in the column and, at a given value of the distribution constant, to a decrease in the mobile phase volume necessary for elution of the solute. This effect creates good conditions for miniaturization of the pump. It is possible to show that the required chromatographic parameters can be attained when using the volume of the liquid displaced from the pump cylinder in one stroke of a small-volume piston. In the described apparatus, moreover, it is possible to suck the mobile phase solution in segments of different composition and so to create conditions of gradient elution without application of either demanding technical elements[2–4] or mixers, and without including further dispersion elements[5,6] in the chromatograph. The cylinder volume used does not exceed the syringe volumes currently used in chromatography. The dependence of the basic chromatographic parameters on the diameter of the column used (d_c) and the elution volume (V_R) can be expressed as follows:

$$n = V_R[0.25\ \pi d_c^2 h d_p \varepsilon_u(1 + k)]^{-1} \tag{1}$$

$$k = V_R[0.25\ \pi d_c^2 L \varepsilon_u]^{-1} - 1 \tag{2}$$

$$L = V_R[0.25\ \pi d_c^2 \varepsilon_u(1 + k)]^{-1} \tag{3}$$

where n is number of theoretical plates, k the capacity ratio, L the column length, h the reduced plate, d_p the diameter of the sorbent particle and ε_u the porosity corresponding to the fraction of the sorbent bed through which the mobile phase flows.

TABLE I

EXAMPLE OF SOME CHROMATOGRAPHIC PARAMETERS ACHIEVABLE WITH ONE STROKE OF THE INJECTION PISTON

Injection cylinder volume: 100 μl.

Parameter	*Chromatographic column I.D. (mm)*			
	0.2	*0.5*	*1*	*2*
Number of theoretical plates (n)*	$9.1 \cdot 10^4$	$1.5 \cdot 10^4$	$3.6 \cdot 10^3$	$9.1 \cdot 10^2$
Column length mm (L)*	900	145	36	9
Capacity ratio (k)**	30	4.7	1.1	0.2

* $k = 4$, $h = 2$, $d_p = 5$ μm.
** $L = 150$ mm.

Table I shows an example of the parameters achievable, provided that the retention volume is constant ($V_R = 100$ μl). It is also evident from Table I that for sufficiently small diameters of the column, *e.g.*, $d_c = 0.2$ mm, the reached parameters are sufficient for most routine analyses.

ROLE OF THE SAMPLE VOLUME

A decrease in the column dimensions leads to a decrease in the column sorption capacity which, in most instances, is a fundamental defect of microcolumn chromatography. A marked disproportion appears between the very small retention volume, V_R, of the solute and the technically possible volume of the sample injected, V_S. The requirement for the amount of the sample injected not to exceed the column sorption capacity is expressed by a known relationship [*e.g.*, ref. 7] from which the volume injected can be expressed as a function of the retention volume:

$$V_S = aV_R\sqrt{\frac{hd_p}{L}} = aV_R/\sqrt{n} \tag{4}$$

where a is a proportionality constant.

The maximum admissible volumes injected calculated in such a way vary, for the above-mentioned conditions, in the approximate range 10^{-2}–10^{-7} μl. It is evident that the splitless injection of measured and reproducible volumes of this magnitude is not technically possible at present. To make use of chromatography with small retention volumes, it is necessary to inject substantially larger volumes of the sample without reducing the efficiency of the chromatographic system.

The only solution seems to be peak focusing[8–11] on the chromatographic micro-column[12–16]. The degree of peak focusing and also the maximum volume to be injected can be expressed as a function of the difference between the capacity ratio

of the solute, k_S, in the matrix of the injected sample volume, V_S, and the capacity ratio of a given solute in the mobile phase, k. If the volume injected does not differ too much from the retention volume, the process of injection can be considered as chromatography with a stepwise gradient[17] and the adjusted retention volume of the solute, V'_R, can be expressed as follows:

$$V'_R = V_S \frac{k_S - k}{k_S} + kV_M \tag{5}$$

where V_M is the dead volume of the column.

When $k_S \approx k$, the maximum magnitude of V_S is limited according to eqn. 4. When $k_S \gg k$, eqn. 5 changes to

$$V'_R = V_S + kV_M \tag{6}$$

Under such circumstances, the sample can be injected in a volume that exceeds many-fold the column dead volume. From a general point of view, the retention of the solute in liquid chromatography is always influenced by the volume of the injected sample, the composition of which differs from the mobile phase composition. As a consequence in the beginning sections of the column the solute is eluted by means of a liquid with a composition different from the mobile phase composition. The influence of the sample injected on V_R decreases with decreasing V_S and with increasing V_M. If L, k_S and k are taken as constants, then the influence of the sample volume injected decreases with the increasing diameter of the column, *i.e.*, with increasing V_M. Eqns. 5 and 6 show that the magnitude of the sample injected influenced not only the column efficiency as eqn. 4 indicates, but also the retention characteristics of the solute.

It follows from the above that it is useful to use gradient techniques for focusing the peaks of separated components. The apparatus described, with a simple device for the injection of samples of different volumes with the possibilities of changing the mobile phase composition and gradient formation, makes trace analysis easier.

CHROMATOGRAPH

A schematic representation of the chromatograph is shown in Fig. 1. It consists of a pump, a column and a detector. The pump integrates three functions: mobile phase delivery, sample injection and generation of gradients of the mobile phase composition. It consists of two basic parts: a syringe with the volume of 100 μl (13) and a liquid distribution block (11). To move the syringe plunger, a geared stepping motor (an LD 2 linear injector manufactured by Development Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia) is used. A needle (12) connected to the syringe protrudes into the liquid distribution block (11). It passes through two seals; the first (10) seals the column and the other (2) contains the inlets of mobile phases and the sample (1). The side opening bored in the needle (diameter 0.15 mm) can be adjusted in such a way that it is connected with one of the inlets (1) or it leads via a coupling (2 mm × 0.15 mm I.D.) to the column. The column used

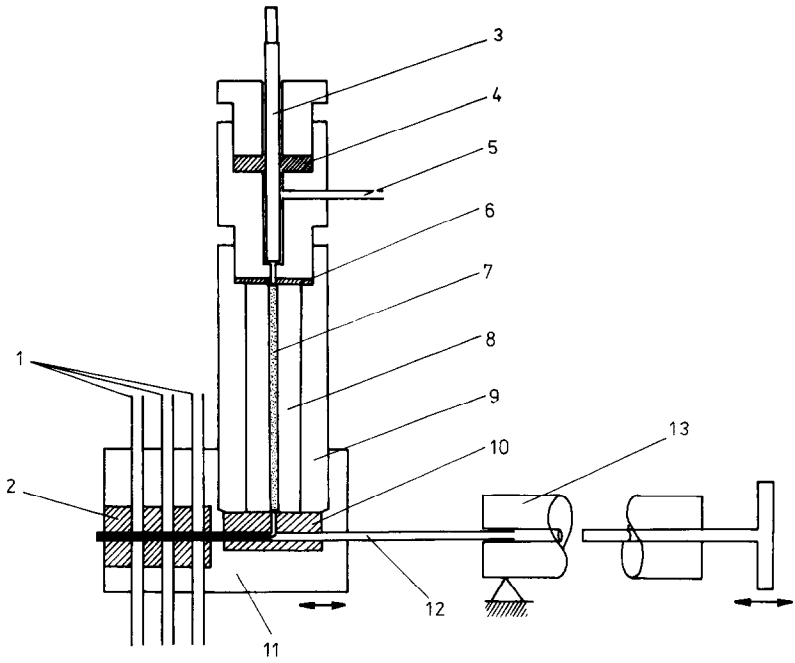

Fig. 1. Design of the micro-chromatograph. 1 = Mobile phase and sample inlets; 2 = PTFE seals for inlets; 3 = electrochemical detector electrode; 4 = silicone rubber seal; 5 = mobile phase waste; 6 = PTFE seal; 7 = column packing; 8 = glass column (30 mm × 0.7 mm I.D. × 7 mm O.D.); 9 = metal jacket of the column; 10 = combined seal of the column and the injection needle (with a 2 mm × 0.15 mm I.D. coupling); 11 = liquid distribution block; 12 = injection needle (0.3 mm I.D., 0.5 mm O.D.) provided with a side outlet (0.15 mm I.D.) and closed at the end; 13 = syringe of volume 100 μl.

was of 30 mm × 0.7 mm I.D., packed with Silasorb SPH C_{18} (Lachema, Brno, Czechoslovakia) with a particle size d_p = 7.5 μm. A suspension of the sorbent in carbon tetrachloride was used for packing. An electrochemical detector[12–14] is connected to the column outlet. The mobile phase flows from the column through the coupling (3 mm × 0.15 mm I.D.) and washes the frontal part of the cross-section of a Pt wire with a diameter of 0.5 mm functioning as a working electrode. The detector volume, including the inlet, is less than 60 nl.

The chromatograph functions in the following way. The mobile phase is sucked into the syringe and then the opening in the needle changes its position and the necessary volume of the sample is sucked up. The opening in the needle then moves to the inlet of the column and the contents of the needle and of the syringe are forced out by means of the plunger through the column into the detector. Under the given conditions, it was possible to inject sample volumes in the range 0.05–2 μl. Volumes smaller than 0.05 μl cannot be injected with sufficient accuracy, and with volumes above 2 μl the sample starts to fill the syringe with a substantially larger diameter and is dispersed. When injecting large sample volumes (up to 100 μl), in trace analysis, a part or the whole of the volume of the syringe can be used and simultaneously one of the previously described[13,15,16] peak focusing techniques should be applied. If it is necessary for successful performance of the analysis to use a gradient of the mobile phase composition, it is possible to proceed in two ways. After carrying out the

isocratic analysis (displacement of the syringe volume), a stronger mobile phase is sucked after moving the opening in the needle, then the opening in the needle moves again to the column inlet and the analysis proceeds. The other possibility is represented by forming a gradient of the mobile phase of a pantile character in the syringe by means of successive sucking up of stronger and weaker mobile phases. In such a way the gradient for the separation of dansyl derivatives of amino acids in Fig. 4 was formed. It is evident that the whole system is very suitable for microprocessor control.

EXPERIMENTAL

The apparatus was designed and manufactured in such a way that the extra-column volume was reduced to minimum and did not influence the efficiency of the chromatographic separation. The efficiency of the system was measured using azo dyes as solutes with different capacity ratios (Table II). The azo dyes were dissolved in the mobile phase, which eliminated the effect of peak focusing at the beginning of the chromatographic column. The heights equivalent to a theoretical plate obtained under such circumstances are dependent not only on the dispersion of the solute in the detector but also on the contribution of the injection system to the sample dispersion. The dead volume of the column used was 8 μl and as a consequence any non-ideal conditions in the injection system can play an important role. The fact that the solute is displaced from the injection needle at right angle to the column and that the diameter of the capillaries used changes from 0.3 to 0.15 mm led to experimental verification of the dispersion of the injected pulse of solute concentration in the injector.

The electrochemical detector was connected directly to the injector and the form and volume of pulses of the solute concentration were measured under different conditions of injection. The volume between the side opening of the injection needle (12) (Fig. 1) and the electrode did not exceed 100 nl. The form of the pulses is evident from Fig. 2. The volume V_i in which the injected sample of volume V_S is transported to the detector is evident from Table III. The volume was measured as an intersection of the tangent of the descending part of the peak with the axis of the mobile phase volume. In comparison with similar studies carried out previously[18–20] on chromatographs designed for work with normal columns, the results acquired in this study are very satisfactory. We conclude that this is due to the fact that, in the course of injection, the flow of the liquid in the injection needle turns. The concentration gradient that appears as a consequence of the profile of the liquid flow in the injection

TABLE II

EXAMPLE OF COLUMN EFFICIENCY

Conditions of analysis: F_m = 0.15 μl s^{-1}; u = 0.65 mm s^{-1}; Mobile phase, acetonitrile–water (70:30).

Solute	*k*	*H* (μm)	*h*
4-Aminoazobenzene	1.5	25	3.6
2-Aminoazotoluene	3.5	18	2.6
N,N-Dimethyl-4-aminoazobenzene	5.5	18	2.6

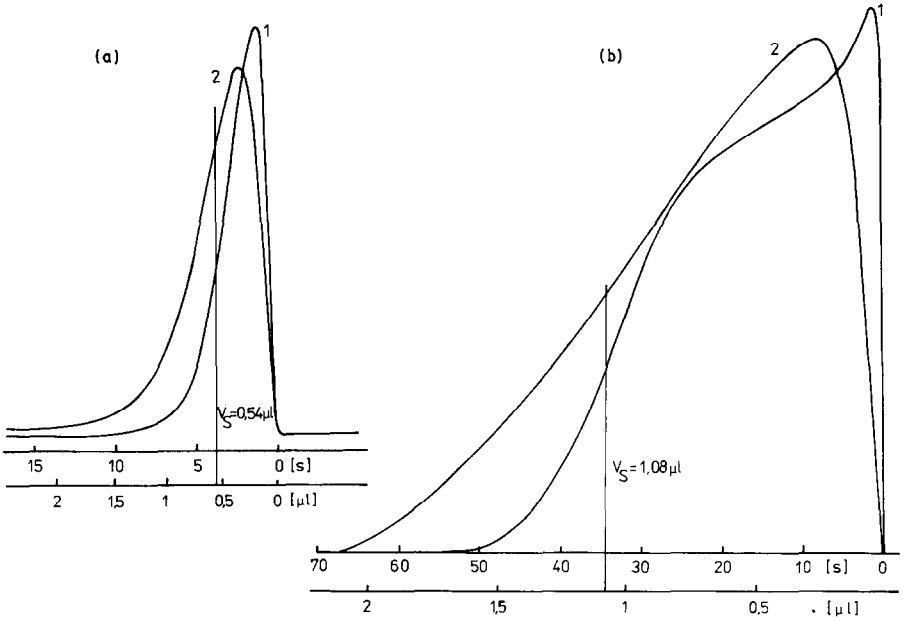

Fig. 2. Examples of influence of sample suction velocity on peak dispersion. Mobile phase: water. Sample: 1% sodium nitrite in water. (a) Volume injected: 0.54 µl. Suction velocity: (1) 0.05 µl s^{-1}; (2) 0.5 µl s^{-1}. Displacement: 0.15 µl s^{-1}. (b) Volume injected: 1.08 µl. Suction velocity: (1) 0.1 µl s^{-1}; (2) 1 µl s^{-1}. Displacement: 0.03 µl s^{-1}.

TABLE III

INFLUENCE OF THE VELOCITY OF SUCTION AND DISPLACEMENT OF THE SAMPLE ON PEAK DISPERSION

F_m = Sample displacement velocity, identical with mobile phase flow-rate; V_S = sample volume; V_i = volume in which V_S is transported to the detector (volume injected).

F_m (µl s^{-1})	V_S (µl)	V_i (µl)	
		Slow suction (0.1 µl/s)	*Rapid suction (1 µl/s)*
0.303	0.27	0.7	1.0
	0.54	1.3	1.5
	1.08	1.7	1.9
0.154	0.27	0.8	0.8
	0.54	1.1	1.2
	1.08	2.1	2.3
0.077	0.27	0.6	0.7
	1.08	1.5	1.8
0.031	0.27	0.5	0.6
	0.54	0.8	1.0
	1.08	1.4	1.8

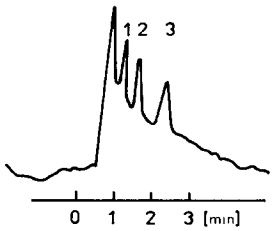

Fig. 3. Enrichment and analysis of trace amounts of chlorophenols. 1 = 4-Chlorophenol; 2 = 2,4-dichlorophenol; 3 = 2,4,5-trichlorophenol (2.5 ng each). A 500-μl volume of aqueous solution of chlorophenols (5 ppb) was sqeezed through the column and chlorophenols were retained at the beginning of the column and then eluted by the mobile phase (acetonitrile–water, 70:30). Column: 30 mm × 0.7 mm I.D. Sorbent: Silasorb SPH C_{18} (7.5 μm).

needle decreases. During the currently used method of injection by means of sampling valves, the flow stops, even if only for a short time, and consequently a pressure difference appears on the valve. After finishing the injection, the difference is compensated for and the volume injected is displaced on the column at an increased velocity. The chromatograph enables one to control both the suction velocity and the speed of displacement of the sample on the column. It is evident from Fig. 2 and Table III that it is possible, using this method, to reduce the peak broadening substantially during injection.

Both the form and the volume in which the concentration pulse of the solute is transported on the column are influenced. At a low flow-rate of the mobile phase, *i.e.*, with slow displacement of the liquid from the injection device and slow suction of the sample to the injection device, the concentration pulse is closer to the ideal rectangular form than it is at higher speeds. It is further evident from Table III that the volume in which the sample is injected on to the column is only 1.3 times higher than the sample volume.

In the described device, the volume of the sample injected can be smoothly changed. For the range 0.18–0.54 μl, the calibration dependences were linear. After evaluation by the least-squares method, a correlation coefficient of $r = 0.995$ was found, measured with the electrochemical detector. The possibility of injecting large volumes of sample can be used for trace analysis. Using as an example the analysis of an aqueous solution of chlorophenol at a concentration of 5 ppb, the possibility of injecting 500 μl of the sample is demonstrated. Under such conditions, the solute is injected in the non-eluting solvent (water) and is enriched directly on the column. In the resulting chromatogram (Fig. 3), the individual chlorophenols are detected by means of the electrochemical detector and they represent amounts of 2.5 ng of each component.

The short micro-column used is sufficiently effective for the analysis of fairly complex mixtures. A mobile phase gradient was used for the separation and determination of thirteen dansyl derivatives of amino acids (Fig. 4). The overall analysis was carried out in less than 5 min.

It is evident from the examples mentioned that the miniature apparatus with the small mobile phase volume complies with all the current parameters required for liquid chromatographs. Moreover, the system allows a precise and rapid preparation

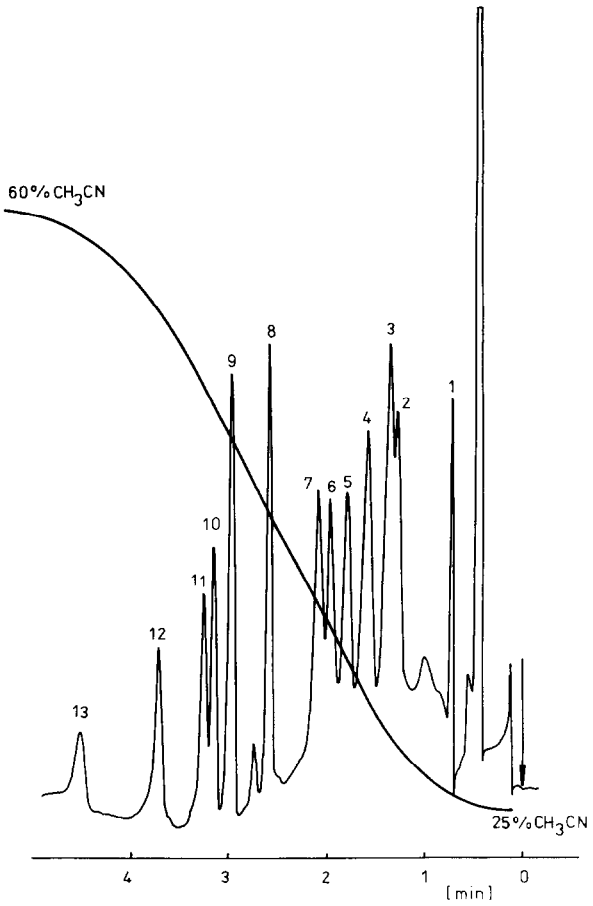

Fig. 4. Gradient elution of dansyl derivatives of amino acids. 1 = Cys a; 2 = Asn; 3 = Glu; 4 = Ser; 5 = Glu a; 6 = OH-Pro; 7 = Thr; 8 = Ala; 9 = Pro; 10 = Phe; 11 = Leu; 12 = di-Lys; 13 = di-Tyr. A 0.5-μl volume of the mixture of dansyl derivatives of amino acids in water ($5 \cdot 10^{-12}$ mole each) was injected on to the column (30 mm × 0.7 mm I.D.) and eluted with a mobile phase gradient from 25% to 60% acetonitrile in 0.01 *M* acetate buffer (pH 3.5).

of the mobile phase and, consequently, a rapid optimization of the chromatographic separation. The injection of a wide range of sample volumes permits both highly effective separations of small samples of high mass sensitivity, and high concentration sensitivity due to the use of enrichment techniques directly on the column. The easy formation of the mobile phase gradient increases the versatility of analytical use. All the above-mentioned qualities allow the apparatus to be used as a tuned chromatographic analyser applicable directly in place of sampling. Also, the fact that more than 3000 analyses were carried out on a 30 mm × 0.7 mm I.D. column packed with a reversed-phase material is considered to be advantageous.

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